

**Title: Methods of Improving the Effectiveness of Transgenic
Plants**

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METHODS OF IMPROVING THE EFFECTIVENESS OF TRANSGENIC PLANTS

5 This application claims benefit of U.S. Provisional Patent Application
Serial No. 60/211,585, filed on June 15, 2000, which is hereby incorporated by
reference in its entirety.

FIELD OF THE INVENTION

10 The present invention relates generally to transgenic plants and
methods of improving the effectiveness of transgenic plants either by topical
application of a hypersensitive response elicitor to the transgenic plant or by
incorporating into the transgenic plant a transgene encoding a hypersensitive response
elicitor.

BACKGROUND OF THE INVENTION

15 Transfer of genes into plants is an approach being used with increasing
frequency to provide useful and advantageous characteristics to crop and ornamental
plants that would be difficult or impossible by traditional breeding methods.
20 Transgenic traits can provide the capacity to synthesize specific compounds including
vaccines, antibodies, pharmaceutical peptides, plastic, or industrial enzymes or
provide improved physical characteristics such as modified fruit ripening, altered
fiber properties, enhanced nutrient or dietary fiber content, herbicide resistance, floral
25 color, or better flavor. Other introduced traits are intended to overcome or minimize
particular agricultural problems, such as environmental stress, or attack by specific
pathogens or pests that prevent maximum yields from being obtained. Transgenic
traits that have been commercialized to date have had very specific and limited
functions. Many other transgenic traits currently being developed for
30 commercialization or being considered for introduction into crops are similarly
limited or specific in their function.

 Environmental factors are an important constraint on the yields
obtained from transgenic as well as non-transgenic crops. Losses in productivity due

to disease and damage caused by pathogens and pests can prevent the full benefit of a transgenic trait from being realized. Since many transgenic traits have no effect on disease or pest resistance, transgenic plants are typically just as susceptible to loss and damage as non-transgenic plants. Transgenic traits designed to confer resistance to pests or disease are, in general, limited in scope — i.e., they are effective only against specific pests or diseases. Such transgenic plants are as vulnerable to non-target pests and diseases as non-transgenic plants. Moreover, the process of introducing a transgenic trait can on occasion result in a crop plant becoming more susceptible to a particular disease. This was observed for some varieties of insect resistant transgenic cotton that lost resistance to a particular fungal pathogen.

Genetically determined inherent growth characteristics of any transgenic plant impose an additional limitation on the potential for benefit to be gained. Transgenic traits being developed for commercialization or that have been commercialized to date do not affect plant growth properties, so efficacy of the traits is restricted by an upper limit on growth even under ideal growing conditions. In some cases it has been observed that the introduction of a transgene conferring a value-added trait can actually cause a reduction in yield. Such a reduction in yield is known as a yield penalty. Yield penalties are tolerated when the value-added trait results in a net economic gain; however, reducing or eliminating the yield penalty would be a clear benefit.

A practical constraint on realizing the maximal benefit from transgenic traits is imposed by the length of time required to develop a transgenic crop to the commercial stage. By the time a transgenic line reaches commercialization, the germplasm used as the starting material may be five or more years old and be at a disadvantage in terms of yield or resistance to specific diseases or pests relative to new germplasms developed in the intervening years. Therefore, it would be desirable to provide an approach that would maximize the benefits of a value-added trait, overcome the yield penalty caused by introduction of a value-added trait, and more rapidly develop a transgenic crop or ornamental lines. To achieve these objectives using existing methods or strategies would be excessively time consuming, technically complex, and without any guarantee of success.

A conventional breeding program is one approach that could be chosen to attempt to obtain a genetic background exhibiting enhanced growth and resistance to diseases and pests into which transgenic traits could be introduced. Unfortunately, achieving even marginal improvements in any one of these characteristics by classical breeding has become increasingly difficult and time consuming as the remaining amount of untapped genetic resources available within a given crop species becomes smaller. There is also no guarantee that this approach is feasible since it is unknown whether achieving useful improvements in all these characteristics simultaneously is possible by conventional breeding.

An alternate approach, at least in principle, would be to introduce into plants, in addition to a gene conferring a desired value-added trait, an array of genes each with a specific resistance or growth enhancement trait to provide an umbrella of resistance and yield improvement effects. A large number of genes have been identified that encode proteins with potential to provide resistance to specific types or classes of pathogens if expressed in transgenic plants. In principle, assembling multiple resistance genes in a transgenic plant could confer resistance to a broad range of pathogens. Such resistance genes, however, would not alter the inherent growth characteristics of the plants. Candidate genes that would serve to enhance overall growth and yield in concert with resistance genes are not obvious. Successfully producing transgenic crops that express arrays of transgenes would be technically complex and require even longer development times than are already needed for generating transgenic plants with a single transgene. Introduction of arrays of transgenes into the same crop plant is an approach yet to be proven in practice.

The use of chemical supplements, including fertilizers and pesticides, to enhance realization of value-added traits is also undesirable due to direct and lingering environmental impact which the chemical supplements can have on water supplies and other organisms in the food chain.

The present invention is directed to overcoming these and other deficiencies in the art.

SUMMARY OF THE INVENTION

One method of the present invention is carried out by providing a plant or plant seed including a transgene conferring a transgenic trait to the plant or a plant grown from the plant seed, and applying to the plant or plant seed a hypersensitive response elicitor protein or polypeptide. According to one embodiment, the applying of the hypersensitive response elicitor is carried out under conditions effective to impart enhanced growth, stress tolerance, disease resistance, or insect resistance to the plant or the plant grown from the plant seed, thereby maximizing the benefit of the transgenic trait to the plant or the plant grown from the plant seed. According to another embodiment, the transgenic trait is associated with a deleterious effect on growth, stress tolerance, disease resistance, or insect resistance in the transgenic plant and the applying of the hypersensitive response elicitor is carried out under conditions effective to impart enhanced growth, stress tolerance, disease resistance, or insect resistance to the plant or the plant grown from the plant seed, thereby overcoming the deleterious effect.

Another method of the present invention is carried out by providing a plant cell, transforming the plant cell with (i) a first DNA molecule encoding a transcript or a protein or polypeptide which confers a trait to a plant grown from the transformed plant cell and (ii) a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide which is different than the protein or polypeptide encoded by the first DNA molecule, the transforming being carried out under conditions effective to produce a transformed plant cell, and then regenerating a transgenic plant from the transformed plant cell. According to one embodiment, transforming with the second DNA molecule imparts enhanced growth, stress tolerance, disease resistance, or insect resistance to the plant, thereby maximizing benefit to the plant of the trait conferred by transforming with the first DNA molecule. According to another embodiment, transforming with the first DNA molecule is accompanied by a deleterious effect on growth, stress tolerance, disease resistance, or insect resistance and transforming with the second DNA molecule overcomes the deleterious effect.

Another aspect of the present invention relates to a transgenic plant including a first DNA molecule encoding a transcript or a protein or polypeptide that confers a trait and a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide different than the protein or polypeptide encoded by the first DNA molecule. Also disclosed is a transgenic plant seed obtained from the transgenic plant of the present invention.

A further aspect of the present invention relates to a system for use in transforming plants with multiple DNA molecules. The system includes a first DNA construct including a first DNA molecule which confers a trait to a host plant and a second DNA construct including a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide. Also disclosed is an expression system including first and second vectors into which are inserted, respectively, the first and second DNA constructs.

A related aspect of the present invention concerns a DNA construct including a first DNA molecule which confers a trait to a host plant and a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide. Also disclosed is an expression system including a vector into which is inserted a DNA construct which includes the first and second DNA molecules.

Yet another aspect of the present invention relates to a transgenic host cell including a first DNA molecule encoding a transcript or a protein or polypeptide that confers a trait to a host plant and a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide which is different than the protein or polypeptide encoded by the first DNA molecule.

The hypersensitive response elicitor, when expressed in or topically applied to transgenic plants, confers a trait of enhanced growth, stress tolerance, broad insect resistance, and broad disease resistance (see WO 96/39802; WO 98/24297; WO 98/32844; and WO 98/37752, which are hereby incorporated by reference in their entirety). By either (i) simultaneously introducing a value-added trait and a trait for hypersensitive response elicitor expression into a plant line or (ii) topically applying a hypersensitive response elicitor to a transgenic plant line expressing a value-added trait, it is possible to obtain a transgenic plant line from which the maximal benefit of the value-added trait can be realized. For example, value-added traits which offer

strong but limited benefits (e.g., resistance to a particular pathogen) can be fully realized either by transforming the plants with a transgene or DNA molecule encoding a hypersensitive response elicitor or applying the hypersensitive response elicitor to the plants, both of which will further enhance the same trait by imparting broad growth enhancement, stress tolerance, disease resistance, and/or insect resistance. Similarly, value-added traits which result in a concomitant yield penalty can be fully realized either by transforming the plants with a transgene or DNA molecule encoding a hypersensitive response elicitor or applying the hypersensitive response elicitor to the plants, both of which will overcome the yield penalty by imparting broad growth enhancement, stress tolerance, disease resistance, and/or insect resistance. When expression is utilized rather than topical application, a transgenic germplasm that expresses a hypersensitive response elicitor (i.e., already has enhanced disease resistance and yield properties beyond what is available from conventional hybrid lines) can be transformed with a transgene conferring a specific value-added trait. The same can be said for subsequent introduction of a transgene coding for hypersensitive response elicitor expression into a transgenic germplasm that already expresses a specific value-added trait. Any of these approaches will likely minimize or eliminate any disadvantages relative to conventional hybrids. Thus, the present invention provides an efficient and simple approach which allows for maximal realization of value-added traits and avoids the short-comings and uncertainties of conventional breeding programs.

DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention is a method carried out by providing a plant or plant seed including a transgene conferring a transgenic trait to the plant or a plant grown from the plant seed, and then applying to the plant or plant seed a hypersensitive response elicitor protein or polypeptide. By applying the hypersensitive response elicitor to the plant or plant seed, as discussed *infra*, enhanced growth, stress tolerance, disease resistance, or insect resistance can be imparted to transgenic plants.

According to one embodiment, the applying of the hypersensitive response elicitor is carried out under conditions effective to impart enhanced growth, stress tolerance, disease resistance, or insect resistance to the plant or the plant grown from the plant seed, thereby maximizing the benefit of the transgenic trait to the plant or the plant grown from the plant seed. For example, when the particular value-added trait relates to specific but limited growth enhancement, stress tolerance, disease resistance, or insect resistance of a transgenic plant, this embodiment relates to providing broad growth enhancement, stress tolerance, disease resistance, or insect resistance that complements the specific but limited value-added trait.

According to another embodiment, the transgenic trait is associated with a deleterious effect on growth, stress tolerance, disease resistance, or insect resistance in the transgenic plant and the applying of the hypersensitive response elicitor is carried out under conditions effective to impart enhanced growth, stress tolerance, disease resistance, or insect resistance to the plant or the plant grown from the plant seed, thereby overcoming the deleterious effect. Thus, this aspect of the present invention is directed to overcoming a yield penalty resulting from a value-added trait.

According to this aspect of the present invention, the effectiveness of a transgenic plant is improved (i.e., maximum benefit is realized or the yield penalty is overcome) following application of a hypersensitive response elicitor protein or polypeptide to either a transgenic plant or a transgenic plant seed from which a plant is grown. The hypersensitive response elicitor protein or polypeptide can be any hypersensitive response elicitor derived from bacterial or fungal sources, although bacterial sources are preferred.

Exemplary hypersensitive response elicitor proteins and polypeptides from bacterial sources include, without limitation, the hypersensitive response elicitors from *Erwinia* species (e.g., *Erwinia amylovora*, *Erwinia chrysanthemi*, *Erwinia stewartii*, *Erwinia carotovora*, etc.), *Pseudomonas* species (e.g., *Pseudomonas syringae*, *Pseudomonas solanacearum*, etc.), and *Xanthomonas* species (e.g., *Xanthomonas campestris*). In addition to hypersensitive response elicitors from these Gram-negative bacteria, it is possible to use elicitors from Gram-positive

bacteria. One example is the hypersensitive response elicitor from *Clavibacter michiganensis* subsp. *sepedonicus*.

Exemplary hypersensitive response elicitor proteins or polypeptides from fungal sources include, without limitation, the hypersensitive response elicitors (i.e., elicitors) from various *Phytophthora* species (e.g., *Phytophthora parasitica*, *Phytophthora cryptogea*, *Phytophthora cinnamomi*, *Phytophthora capsici*, *Phytophthora megasperma*, *Phytophthora citrophthora*, etc.).

The hypersensitive response elicitor protein or polypeptide is derived, preferably, from *Erwinia chrysanthemi*, *Erwinia amylovora*, *Pseudomonas syringae*, or *Pseudomonas solanacearum*.

A hypersensitive response elicitor protein or polypeptide from *Erwinia chrysanthemi* has an amino acid sequence corresponding to SEQ. ID. No. 1 as follows:

15	Met	Gln	Ile	Thr	Ile	Lys	Ala	His	Ile	Gly	Gly	Asp	Leu	Gly	Val	Ser	1	5	10	15
	Gly	Leu	Gly	Ala	Gln	Gly	Leu	Lys	Gly	Leu	Asn	Ser	Ala	Ala	Ser	Ser	20	25	30	
20	Leu	Gly	Ser	Ser	Val	Asp	Lys	Leu	Ser	Ser	Thr	Ile	Asp	Lys	Leu	Thr	35	40	45	
	Ser	Ala	Leu	Thr	Ser	Met	Met	Phe	Gly	Gly	Ala	Leu	Ala	Gln	Gly	Leu	50	55	60	
	Gly	Ala	Ser	Ser	Lys	Gly	Leu	Gly	Met	Ser	Asn	Gln	Leu	Gly	Gln	Ser	65	70	75	80
25	Phe	Gly	Asn	Gly	Ala	Gln	Gly	Ala	Ser	Asn	Leu	Leu	Ser	Val	Pro	Lys	85	90	95	
	Ser	Gly	Gly	Asp	Ala	Leu	Ser	Lys	Met	Phe	Asp	Lys	Ala	Leu	Asp	Asp	100	105	110	
30	Leu	Leu	Gly	His	Asp	Thr	Val	Thr	Lys	Leu	Thr	Asn	Gln	Ser	Asn	Gln	115	120	125	
	Leu	Ala	Asn	Ser	Met	Leu	Asn	Ala	Ser	Gln	Met	Thr	Gln	Gly	Asn	Met	130	135	140	
	Asn	Ala	Phe	Gly	Ser	Gly	Val	Asn	Asn	Ala	Leu	Ser	Ser	Ile	Leu	Gly	145	150	155	160
35	Asn	Gly	Leu	Gly	Gln	Ser	Met	Ser	Gly	Phe	Ser	Gln	Pro	Ser	Leu	Gly	165	170	175	
	Ala	Gly	Gly	Leu	Gln	Gly	Leu	Ser	Gly	Ala	Gly	Ala	Phe	Asn	Gln	Leu	180	185	190	
40	Gly	Asn	Ala	Ile	Gly	Met	Gly	Val	Gly	Gln	Asn	Ala	Ala	Leu	Ser	Ala	195	200	205	

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This hypersensitive response elicitor protein or polypeptide has a molecular weight of about 39 kDa, has a pI of approximately 4.3, and is heat stable at 100°C for at least 10 minutes. This hypersensitive response elicitor protein or polypeptide has substantially no cysteine. The hypersensitive response elicitor protein or polypeptide derived from *Erwinia amylovora* is more fully described in Wei, Z-M., et al., "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992), which is hereby incorporated by reference in its entirety. The DNA molecule encoding this hypersensitive response elicitor protein or polypeptide has a nucleotide sequence corresponding to SEQ. ID. No. 4 as follows:

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aagcttcggc atggcacgtt tgaccgttgg gtcggcaggg tacgtttgaa ttattcataa 60
gaggaatacg ttatgagtct gaatacaagt gggctgggag cgtcaacgat gcaaatttct 120
atcggcgggtg cgggcggaaa taacgggttg ctgggtacca gtcgccagaa tgctgggttg 180
ggtggcaatt ctgcactggg gctgggcggc ggtaatcaaa atgataccgt caatcagctg 240
gctggccttac tcaccggcat gatgatgatg atgagcatga tgggcgggtg tgggctgatg 300
ggcgggtggct taggcgggtg cttaggtaat ggcttgggtg gctcaggtgg cctgggcgaa 360
ggactgtcga acgcgctgaa cgatatgtta ggcgggttcgc tgaacacgct gggctcgaaa 420
ggcggcaaca ataccacttc aacaacaaat tccccgctgg accaggcgct ggggtattaac 480
tcaacgtccc aaaacgacga ttccacctcc ggcacagatt ccacctcaga ctccagcgac 540
ccgatgcagc agctgctgaa gatgttcagc gagataatgc aaagcctgtt tgggtgatgg 600
caagatggca cccagggcag ttctctctggg ggcaagcagc cgaccgaagg cgagcagaac 660
gcctataaaa aaggagtcac tgatgcgctg tcgggcctga tgggtaatgg tctgagccag 720
ctccttggca acgggggact gggaggtggt cagggcggta atgctggcac gggctctgac 780
ggttcgctgc tgggcggcaa agggctgcaa aacctgagcg ggccgggtgga ctaccagcag 840
ttaggtaacg ccgtgggtac cggtatcggt atgaaagcgg gcattcaggc gctgaatgat 900
atcgggtacgc acaggcacag ttcaaccctg tctttcgtca ataaaggcga tcgggcgatg 960
gcgaaggaaa tcggtcagtt catggaccag tatcctgagg tgtttggcaa gccgcagtac 1020
cagaaaggcc cgggtcagga ggtgaaaacc gatgacaaat catgggcaaa agcactgagc 1080
aagccagatg acgacggaat gacaccagcc agtatggagc agttcaacaa agccaagggc 1140
atgatcaaaa ggcccatggc gggtgatacc ggcaacggca acctgcaggc acgcgggtgcc 1200
ggtggttctt cgctgggtat tgatgccatg atggccggtg atgccattaa caatatggca 1260
cttggaagc tgggcgcggc ttaagctt 1288
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The above nucleotide and amino acid sequences are disclosed and further described in U.S. Patent No. 5,849,868 to Beer et al. and U.S. Patent No. 5,776,889 to Wei et al., which are hereby incorporated by reference in their entirety.

Another hypersensitive response elicitor protein or polypeptide derived from *Erwinia amylovora* has an amino acid sequence corresponding to SEQ. ID. No. 5 as follows:

	Met	Ser	Ile	Leu	Thr	Leu	Asn	Asn	Asn	Thr	Ser	Ser	Ser	Pro	Gly	Leu	
	1				5					10					15		
10	Phe	Gln	Ser	Gly	Gly	Asp	Asn	Gly	Leu	Gly	Gly	His	Asn	Ala	Asn	Ser	
				20					25					30			
	Ala	Leu	Gly	Gln	Gln	Pro	Ile	Asp	Arg	Gln	Thr	Ile	Glu	Gln	Met	Ala	
			35					40					45				
15	Gln	Leu	Leu	Ala	Glu	Leu	Leu	Lys	Ser	Leu	Leu	Ser	Pro	Gln	Ser	Gly	
		50					55					60					
	Asn	Ala	Ala	Thr	Gly	Ala	Gly	Gly	Asn	Asp	Gln	Thr	Thr	Gly	Val	Gly	
	65					70				75					80		
	Asn	Ala	Gly	Gly	Leu	Asn	Gly	Arg	Lys	Gly	Thr	Ala	Gly	Thr	Thr	Pro	
					85					90				95			
20	Gln	Ser	Asp	Ser	Gln	Asn	Met	Leu	Ser	Glu	Met	Gly	Asn	Asn	Gly	Leu	
				100					105					110			
	Asp	Gln	Ala	Ile	Thr	Pro	Asp	Gly	Gln	Gly	Gly	Gly	Gln	Ile	Gly	Asp	
			115					120					125				
25	Asn	Pro	Leu	Leu	Lys	Ala	Met	Leu	Lys	Leu	Ile	Ala	Arg	Met	Met	Asp	
		130					135					140					
	Gly	Gln	Ser	Asp	Gln	Phe	Gly	Gln	Pro	Gly	Thr	Gly	Asn	Asn	Ser	Ala	
	145					150					155				160		
	Ser	Ser	Gly	Thr	Ser	Ser	Ser	Gly	Gly	Ser	Pro	Phe	Asn	Asp	Leu	Ser	
					165					170					175		
30	Gly	Gly	Lys	Ala	Pro	Ser	Gly	Asn	Ser	Pro	Ser	Gly	Asn	Tyr	Ser	Pro	
				180					185					190			
	Val	Ser	Thr	Phe	Ser	Pro	Pro	Ser	Thr	Pro	Thr	Ser	Pro	Thr	Ser	Pro	
			195					200					205				
35	Leu	Asp	Phe	Pro	Ser	Ser	Pro	Thr	Lys	Ala	Ala	Gly	Gly	Ser	Thr	Pro	
		210					215					220					
	Val	Thr	Asp	His	Pro	Asp	Pro	Val	Gly	Ser	Ala	Gly	Ile	Gly	Ala	Gly	
	225					230					235				240		
	Asn	Ser	Val	Ala	Phe	Thr	Ser	Ala	Gly	Ala	Asn	Gln	Thr	Val	Leu	His	
				245						250					255		
40	Asp	Thr	Ile	Thr	Val	Lys	Ala	Gly	Gln	Val	Phe	Asp	Gly	Lys	Gly	Gln	
				260					265					270			
	Thr	Phe	Thr	Ala	Gly	Ser	Glu	Leu	Gly	Asp	Gly	Gly	Gln	Ser	Glu	Asn	
			275					280					285				

Gln Lys Pro Leu Phe Ile Leu Glu Asp Gly Ala Ser Leu Lys Asn Val
 290 295 300
 Thr Met Gly Asp Asp Gly Ala Asp Gly Ile His Leu Tyr Gly Asp Ala
 305 310 315 320
 5 Lys Ile Asp Asn Leu His Val Thr Asn Val Gly Glu Asp Ala Ile Thr
 325 330 335
 Val Lys Pro Asn Ser Ala Gly Lys Lys Ser His Val Glu Ile Thr Asn
 340 345 350
 10 Ser Ser Phe Glu His Ala Ser Asp Lys Ile Leu Gln Leu Asn Ala Asp
 355 360 365
 Thr Asn Leu Ser Val Asp Asn Val Lys Ala Lys Asp Phe Gly Thr Phe
 370 375 380
 Val Arg Thr Asn Gly Gly Gln Gln Gly Asn Trp Asp Leu Asn Leu Ser
 385 390 395 400
 15 His Ile Ser Ala Glu Asp Gly Lys Phe Ser Phe Val Lys Ser Asp Ser
 405 410 415
 Glu Gly Leu Asn Val Asn Thr Ser Asp Ile Ser Leu Gly Asp Val Glu
 420 425 430
 20 Asn His Tyr Lys Val Pro Met Ser Ala Asn Leu Lys Val Ala Glu
 435 440 445

This protein or polypeptide is acidic, rich in glycine and serine, and lacks cysteine. It is also heat stable, protease sensitive, and suppressed by inhibitors of plant metabolism. The protein or polypeptide of the present invention has a predicted molecular size of ca. 4.5 kDa. The DNA molecule encoding this hypersensitive response elicitor protein or polypeptide has a nucleotide sequence corresponding to SEQ. ID. No. 6 as follows:

30 atgtcaattc ttacgcttaa caacaatacc tcgtcctcgc cgggtctggt ccagtcceggg 60
 ggggacaacg ggcttggtgg tcataatgca aattctgcgt tggggcaaca acccatcgat 120
 cggcaaacca ttgagcaa at ggctcaatta ttggcggaac tggttaaagtc actgctatcg 180
 ccacaatcag gtaatgcggc aaccggagcc ggtggcaatg accagactac aggagttggt 240
 aacgctggcg gcctgaacgg acgaaaaggc acagcaggaa ccaactccgca gtctgacagt 300
 35 cagaacatgc tgagtgcgat gggcaacaac gggctggatc aggccatcac gccgatggc 360
 cagggcggcg ggcagatcgg cgataatcct ttactgaaag ccatgctgaa gcttattgca 420
 cgcgatgatgg acggccaaag cgatcagttt ggccaacctg gtacgggcaa caacagtgcc 480
 tcttcgggta cttcttcac tggcggttcc ccttttaacg atctatcagg ggggaaggcc 540
 ccttcgggca actccccttc cggcaactac tctcccgta gtaccttctc acccccatcc 600
 40 acgccaacgt cccctacctc accgcttgat ttcccttctt ctcccaccaa agcagccggg 660

ggcagcacgc cggtaaccga tcatacctgac cctgttggtgta ggcgcccgcgga 720
aattcgggtgg ccttcaccag cgcgggcgct aatcagacgg tgctgcatga caccattacc 780
gtgaaaagcgg gtcaggtggt tgatggcaaa ggacaaacct tcaccgccgg ttcagaatta 840
ggcgatggcg gccagtctga aaaccagaaa ccgctgttta tactggaaga cggtgccagc 900
5 ctgaaaaacg tcaccatggg cgacgacggg gcggatggta ttcattctta cggatgatgcc 960
aaaatagaca atctgcacgt caccaacgtg ggtgaggacg cgattaccgt taagccaaac 1020
agcgccgggca aaaaatccca cgttgaaatc actaacagtt ccttcgagca cgctctgac 1080
aagatcctgc agctgaatgc cgataactaac ctgagcggtg acaacgtgaa ggccaaagac 1140
tttggtactt ttgtacgcac taacggcggt caacagggtg actgggatct gaatctgagc 1200
10 catatcagcg cagaagacgg taagttctcg ttcgttaaaa gcgatagcga ggggctaaac 1260
gtcaatacca gtgatatctc actgggtgat gttgaaaacc actacaaagt gccgatgtcc 1320
gccaacctga aggtggctga atga 1344

15 The above nucleotide and amino acid sequences are disclosed and further described in
PCT Application Publication No. WO 99/07208 to Kim et al., which is hereby
incorporated by reference in its entirety.

20 A hypersensitive response elicitor protein or polypeptide derived from
Pseudomonas syringae has an amino acid sequence corresponding to SEQ. ID. No. 7
as follows:

Met Gln Ser Leu Ser Leu Asn Ser Ser Ser Leu Gln Thr Pro Ala Met
1 5 10 15
Ala Leu Val Leu Val Arg Pro Glu Ala Glu Thr Thr Gly Ser Thr Ser
25 20 25 30
Ser Lys Ala Leu Gln Glu Val Val Val Lys Leu Ala Glu Glu Leu Met
35 35 40 45
Arg Asn Gly Gln Leu Asp Asp Ser Ser Pro Leu Gly Lys Leu Leu Ala
50 55 60
30 Lys Ser Met Ala Ala Asp Gly Lys Ala Gly Gly Gly Ile Glu Asp Val
65 70 75 80
Ile Ala Ala Leu Asp Lys Leu Ile His Glu Lys Leu Gly Asp Asn Phe
85 90 95
Gly Ala Ser Ala Asp Ser Ala Ser Gly Thr Gly Gln Gln Asp Leu Met
35 100 105 110
Thr Gln Val Leu Asn Gly Leu Ala Lys Ser Met Leu Asp Asp Leu Leu
115 120 125
Thr Lys Gln Asp Gly Gly Thr Ser Phe Ser Glu Asp Asp Met Pro Met
130 135 140

	Leu	Asn	Lys	Ile	Ala	Gln	Phe	Met	Asp	Asp	Asn	Pro	Ala	Gln	Phe	Pro
	145					150					155					160
	Lys	Pro	Asp	Ser	Gly	Ser	Trp	Val	Asn	Glu	Leu	Lys	Glu	Asp	Asn	Phe
					165					170					175	
5	Leu	Asp	Gly	Asp	Glu	Thr	Ala	Ala	Phe	Arg	Ser	Ala	Leu	Asp	Ile	Ile
				180					185					190		
	Gly	Gln	Gln	Leu	Gly	Asn	Gln	Gln	Ser	Asp	Ala	Gly	Ser	Leu	Ala	Gly
			195				200						205			
10	Thr	Gly	Gly	Gly	Leu	Gly	Thr	Pro	Ser	Ser	Phe	Ser	Asn	Asn	Ser	Ser
	210						215					220				
	Val	Met	Gly	Asp	Pro	Leu	Ile	Asp	Ala	Asn	Thr	Gly	Pro	Gly	Asp	Ser
	225					230					235					240
	Gly	Asn	Thr	Arg	Gly	Glu	Ala	Gly	Gln	Leu	Ile	Gly	Glu	Leu	Ile	Asp
					245					250					255	
15	Arg	Gly	Leu	Gln	Ser	Val	Leu	Ala	Gly	Gly	Gly	Leu	Gly	Thr	Pro	Val
				260					265					270		
	Asn	Thr	Pro	Gln	Thr	Gly	Thr	Ser	Ala	Asn	Gly	Gly	Gln	Ser	Ala	Gln
			275					280					285			
20	Asp	Leu	Asp	Gln	Leu	Leu	Gly	Gly	Leu	Leu	Leu	Lys	Gly	Leu	Glu	Ala
	290						295					300				
	Thr	Leu	Lys	Asp	Ala	Gly	Gln	Thr	Gly	Thr	Asp	Val	Gln	Ser	Ser	Ala
	305					310					315					320
	Ala	Gln	Ile	Ala	Thr	Leu	Leu	Val	Ser	Thr	Leu	Leu	Gln	Gly	Thr	Arg
					325					330					335	
25	Asn	Gln	Ala	Ala	Ala											
					340											

This hypersensitive response elicitor protein or polypeptide has a molecular weight of 34-35 kDa. It is rich in glycine (about 13.5%) and lacks cysteine and tyrosine.

Further information about the hypersensitive response elicitor derived from *Pseudomonas syringae* is found in He, S. Y., et al., "*Pseudomonas syringae* pv. *syringae* Harpin_{PSS}: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993), which is hereby incorporated by reference in its entirety. The DNA molecule encoding this hypersensitive response elicitor from *Pseudomonas syringae* has a nucleotide sequence corresponding to SEQ. ID. No. 8 as follows:

atgcagagtc	tcaagtcttaa	cagcagctcg	ctgcaaacc	cggcaatggc	ccttgctctg	60
gtacgtcctg	aagccgagac	gactggcagt	acgtcgagca	aggcgcttca	ggaagttgtc	120
gtgaagctgg	ccgaggaact	gatgcgcaat	ggcgaactcg	acgacagctc	gccattggga	180
aaactgttgg	ccaagtcgat	ggccgcagat	ggcaaggcgg	gcggcggtat	tgaggatgtc	240

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atcgctgcg tggacaagct gatccatgaa aagctcgggtg acaacttcgg cgcgtctgcg 300
gacagcgctt cgggtaccgg acagcaggac ctgatgactc aggtgctcaa tggcctggcc 360
aagtcgatgc tcgatgatct tctgaccaag caggatggcg ggacaagctt ctccgaagac 420
gatatgccga tgctgaacaa gatcgcgcag ttcattggatg acaatcccgc acagtttccc 480
5 aagccggact cgggctcctg ggtgaacgaa ctcaaggaag acaacttcct tgatggcgac 540
gaaacggctg cgttcctgctt ggcactcgac atcattggcc agcaactggg taatcagcag 600
agtgacgctg gcagtctggc agggacgggt ggaggtctgg gcactccgag cagtttttcc 660
aacaactcgt cctgatggg tgatccgctg atcgacgcca ataccgggtcc cggtgacagc 720
ggcaataccc gtggtgaagc ggggcaactg atcggcgagc ttatcgaccg tggcctgcaa 780
10 tcggtattgg cgggtggtgg actgggcaca cccgtaaaca ccccgagac cggtagctcg 840
gcgaatggcg gacagtcgc tcaggatctt gatcagttgc tgggcggctt gctgctcaag 900
ggcctggagg caacgctcaa ggatgccggg caaacaggca cgcagtgca gtcagcgct 960
gcgcaaatcg ccaccttgct ggtcagtaag ctgctgcaag gcaccgcaa tcaggctgca 1020
gcctga 1026

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The above nucleotide and amino acid sequences are disclosed and further described in U.S. Patent No. 5,708,139 to Collmer et al. and U.S. Patent No. 5,776,889 to Wei et al., which are hereby incorporated by reference in their entirety.

Another hypersensitive response elicitor protein or polypeptide derived from *Pseudomonas syringae* has an amino acid sequence corresponding to SEQ. ID. No. 9 as follows:

```

Met Ser Ile Gly Ile Thr Pro Arg Pro Gln Gln Thr Thr Thr Pro Leu
1           5           10           15
25 Asp Phe Ser Ala Leu Ser Gly Lys Ser Pro Gln Pro Asn Thr Phe Gly
           20           25           30
Glu Gln Asn Thr Gln Gln Ala Ile Asp Pro Ser Ala Leu Leu Phe Gly
           35           40           45
30 Ser Asp Thr Gln Lys Asp Val Asn Phe Gly Thr Pro Asp Ser Thr Val
           50           55           60
Gln Asn Pro Gln Asp Ala Ser Lys Pro Asn Asp Ser Gln Ser Asn Ile
65           70           75           80
Ala Lys Leu Ile Ser Ala Leu Ile Met Ser Leu Leu Gln Met Leu Thr
           85           90           95
35 Asn Ser Asn Lys Lys Gln Asp Thr Asn Gln Glu Gln Pro Asp Ser Gln
           100          105          110
Ala Pro Phe Gln Asn Asn Gly Gly Leu Gly Thr Pro Ser Ala Asp Ser
115          120          125

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	Gly	Gly	Gly	Gly	Thr	Pro	Asp	Ala	Thr	Gly	Gly	Gly	Gly	Gly	Asp	Thr	
	130						135					140					
	Pro	Ser	Ala	Thr	Gly	Gly	Gly	Gly	Gly	Asp	Thr	Pro	Thr	Ala	Thr	Gly	
	145					150				155						160	
5	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Thr	Pro	Thr	Ala	Thr	Gly	Gly	Gly	
					165					170						175	
	Ser	Gly	Gly	Thr	Pro	Thr	Ala	Thr	Gly	Gly	Gly	Glu	Gly	Gly	Val	Thr	
				180					185						190		
10	Pro	Gln	Ile	Thr	Pro	Gln	Leu	Ala	Asn	Pro	Asn	Arg	Thr	Ser	Gly	Thr	
			195					200					205				
	Gly	Ser	Val	Ser	Asp	Thr	Ala	Gly	Ser	Thr	Glu	Gln	Ala	Gly	Lys	Ile	
		210					215					220					
	Asn	Val	Val	Lys	Asp	Thr	Ile	Lys	Val	Gly	Ala	Gly	Glu	Val	Phe	Asp	
	225					230					235					240	
15	Gly	His	Gly	Ala	Thr	Phe	Thr	Ala	Asp	Lys	Ser	Met	Gly	Asn	Gly	Asp	
					245					250					255		
	Gln	Gly	Glu	Asn	Gln	Lys	Pro	Met	Phe	Glu	Leu	Ala	Glu	Gly	Ala	Thr	
				260					265					270			
20	Leu	Lys	Asn	Val	Asn	Leu	Gly	Glu	Asn	Glu	Val	Asp	Gly	Ile	His	Val	
			275					280					285				
	Lys	Ala	Lys	Asn	Ala	Gln	Glu	Val	Thr	Ile	Asp	Asn	Val	His	Ala	Gln	
		290					295					300					
	Asn	Val	Gly	Glu	Asp	Leu	Ile	Thr	Val	Lys	Gly	Glu	Gly	Gly	Ala	Ala	
	305					310					315					320	
25	Val	Thr	Asn	Leu	Asn	Ile	Lys	Asn	Ser	Ser	Ala	Lys	Gly	Ala	Asp	Asp	
					325				330						335		
	Lys	Val	Val	Gln	Leu	Asn	Ala	Asn	Thr	His	Leu	Lys	Ile	Asp	Asn	Phe	
				340					345					350			
30	Lys	Ala	Asp	Asp	Phe	Gly	Thr	Met	Val	Arg	Thr	Asn	Gly	Gly	Lys	Gln	
			355					360					365				
	Phe	Asp	Asp	Met	Ser	Ile	Glu	Leu	Asn	Gly	Ile	Glu	Ala	Asn	His	Gly	
		370					375					380					
	Lys	Phe	Ala	Leu	Val	Lys	Ser	Asp	Ser	Asp	Asp	Leu	Lys	Leu	Ala	Thr	
	385					390				395						400	
35	Gly	Asn	Ile	Ala	Met	Thr	Asp	Val	Lys	His	Ala	Tyr	Asp	Lys	Thr	Gln	
					405					410					415		
	Ala	Ser	Thr	Gln	His	Thr	Glu	Leu									
					420												

This protein or polypeptide is acidic, glycine-rich, lacks cysteine, and is deficient in aromatic amino acids. The DNA molecule encoding this hypersensitive response elicitor from *Pseudomonas syringae* has a nucleotide sequence corresponding to SEQ. ID. No. 10 as follows:

tccacttcgc tgatTTTgaa attggcagat tcatagaaac gttcaggtgt ggaaatcagg 60
ctgagtgcgc agatttcggt gataagggtg tggTactggt cattgttggt catttcaagg 120
cctctgagtg cggTgcggag caataccagt cttcctgctg gcgtgtgcac actgagtcgc 180
5 aggcataggc atttcagttc cttgcgttggt ttgggcatat aaaaaaagga acttttataaa 240
acagtgcagt gagatgccgg caaaacggga accggTcgtt gcgctttgcc actcacttcg 300
agcaagctca accccaaaca tccacatccc tatcgaacgg acagcgatac ggccacttgc 360
tctggTaaac cctggagctg gcgtcggtcc aattgcccac ttagcgaggt aacgcagcat 420
gagcatcggc atcacacccc ggccgcaaca gaccaccacg ccactcgatt tttcggcgct 480
10 aagcggcaag agtcctcaac caaacacgtt cggcgagcag aacactcagc aagcgatcga 540
cccgagtgcA ctgTgttctg gcagcgacac acagaaagac gtcaacttcg gcacgcccga 600
cagcaccgTc cagaatccgc aggacgccag caagcccaac gacagccagt ccaacatcgc 660
taaattgatc agtgcattga tcatgtcgtt gctgcagatg ctcaccaact ccaataaaaa 720
gcaggacacc aatcaggaac agcctgatag ccaggTcctt ttccagaaca acggcgggct 780
15 cggTaccgTc tgggccgata gggggggcgg cggTaccgTc gatgcgacag gtggcggcgg 840
cggTgatacg ccaagcgcaa caggcggTgg cggcggTgat actccgaccg caacaggcgg 900
tggcggcagc ggtggcggcg gcacacccac tgcaacaggt ggcggcagcg gtggcacacc 960
cactgcaaca ggcggTggcg agggTggcgt aacaccgcaa atcactccgc agttggccaa 1020
ccctaaccgt acctcaggta ctggctcggT gtcggacacc gcaggTtcta ccgagcaagc 1080
20 cggcaagatc aatgtggtga aagacaccat caaggTcggc gctggcgaag tctttgacgg 1140
ccacggcgca accttactg ccgacaaatc tatgggtaac ggagaccagg gcgaaaatca 1200
gaagcccatg ttcgagctgg ctgaaggcgc tacgttgaag aatgtgaacc tgggtgagaa 1260
cgaggTcgat ggcattccag tgaaagccaa aaacgTcag gaagtcacca ttgacaacgt 1320
gcatgccag aacgtcggTg aagacctgat tacggTcaaa ggcgagggag gcgcagcggT 1380
25 cactaatctg aacatcaaga acagcagTgc caaaggTgca gacgacaagg ttgtccagct 1440
caacgccaac actcacttga aaatcgacaa cttcaaggcc gacgatttcg gcacgatggT 1500
tcgcaccaac ggtggcaagc agtttTgatga catgagcatc gagctgaacg gcatcgaagc 1560
taaccacggc aagttcgccc tggTgaaaag cgacagtgac gatctgaagc tggcaacggg 1620
caacatcgcc atgaccgacg tcaaacacgc ctacgataaa acccaggcat cgaccaaca 1680
30 caccgagctt tgaatccaga caagtagctt gaaaaaaggg ggtggactc 1729

The above nucleotide and amino acid sequences are disclosed and further described in U.S. Patent No. 6,172,184 to Collmer et al., which is hereby incorporated by reference in its entirety.

A hypersensitive response elicitor protein or polypeptide derived from *Pseudomonas solanacearum* has an amino acid sequence corresponding to SEQ. ID. No. 11 as follows:

5	Met	Ser	Val	Gly	Asn	Ile	Gln	Ser	Pro	Ser	Asn	Leu	Pro	Gly	Leu	Gln	1	5	10	15
	Asn	Leu	Asn	Leu	Asn	Thr	Asn	Thr	Asn	Ser	Gln	Gln	Ser	Gly	Gln	Ser	20	25	30	
10	Val	Gln	Asp	Leu	Ile	Lys	Gln	Val	Glu	Lys	Asp	Ile	Leu	Asn	Ile	Ile	35	40	45	
	Ala	Ala	Leu	Val	Gln	Lys	Ala	Ala	Gln	Ser	Ala	Gly	Gly	Asn	Thr	Gly	50	55	60	
	Asn	Thr	Gly	Asn	Ala	Pro	Ala	Lys	Asp	Gly	Asn	Ala	Asn	Ala	Gly	Ala	65	70	75	80
15	Asn	Asp	Pro	Ser	Lys	Asn	Asp	Pro	Ser	Lys	Ser	Gln	Ala	Pro	Gln	Ser	85	90	95	
	Ala	Asn	Lys	Thr	Gly	Asn	Val	Asp	Asp	Ala	Asn	Asn	Gln	Asp	Pro	Met	100	105	110	
20	Gln	Ala	Leu	Met	Gln	Leu	Leu	Glu	Asp	Leu	Val	Lys	Leu	Leu	Lys	Ala	115	120	125	
	Ala	Leu	His	Met	Gln	Gln	Pro	Gly	Gly	Asn	Asp	Lys	Gly	Asn	Gly	Val	130	135	140	
	Gly	Gly	Ala	Asn	Gly	Ala	Lys	Gly	Ala	Gly	Gly	Gln	Gly	Gly	Leu	Ala	145	150	155	160
25	Glu	Ala	Leu	Gln	Glu	Ile	Glu	Gln	Ile	Leu	Ala	Gln	Leu	Gly	Gly	Gly	165	170	175	
	Gly	Ala	Gly	Ala	Gly	Gly	Ala	Gly	Gly	Gly	Val	Gly	Gly	Ala	Gly	Gly	180	185	190	
30	Ala	Asp	Gly	Gly	Ser	Gly	Ala	Gly	Gly	Ala	Gly	Gly	Ala	Asn	Gly	Ala	195	200	205	
	Asp	Gly	Gly	Asn	Gly	Val	Asn	Gly	Asn	Gln	Ala	Asn	Gly	Pro	Gln	Asn	210	215	220	
	Ala	Gly	Asp	Val	Asn	Gly	Ala	Asn	Gly	Ala	Asp	Asp	Gly	Ser	Glu	Asp	225	230	235	240
35	Gln	Gly	Gly	Leu	Thr	Gly	Val	Leu	Gln	Lys	Leu	Met	Lys	Ile	Leu	Asn	245	250	255	
	Ala	Leu	Val	Gln	Met	Met	Gln	Gln	Gly	Gly	Leu	Gly	Gly	Gly	Asn	Gln	260	265	270	
40	Ala	Gln	Gly	Gly	Ser	Lys	Gly	Ala	Gly	Asn	Ala	Ser	Pro	Ala	Ser	Gly	275	280	285	
	Ala	Asn	Pro	Gly	Ala	Asn	Gln	Pro	Gly	Ser	Ala	Asp	Asp	Gln	Ser	Ser	290	295	300	
45	Gly	Gln	Asn	Asn	Leu	Gln	Ser	Gln	Ile	Met	Asp	Val	Val	Lys	Glu	Val	305	310	315	320

Val Gln Ile Leu Gln Gln Met Leu Ala Ala Gln Asn Gly Gly Ser Gln
325 330 335
Gln Ser Thr Ser Thr Gln Pro Met
340

5

Further information regarding this hypersensitive response elicitor protein or polypeptide derived from *Pseudomonas solanacearum* is set forth in Arlat, M., et al., "PopA1, a Protein which Induces a Hypersensitive-like Response in Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-533 (1994), which is hereby incorporated by reference in its entirety. It is encoded by a DNA molecule from *Pseudomonas solanacearum* having a nucleotide sequence corresponding SEQ. ID. No. 12 as follows:

10

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atgtcagtcg gaaacatcca gagcccgctc aacctcccg gtctgcagaa cctgaacctc 60
aacaccaaca ccaacagcca gcaatcgggc cagtccgtgc aagacctgat caagcaggtc 120
gagaaggaca tcctcaacat catcgcagcc ctctgtcaga aggccgcaca gtcggcgggc 180
ggcaacaccg gtaacaccgg caacgcgccg gcgaaggacg gcaatgccaa cgcgggcgcc 240
aacgacccga gcaagaacga cccgagcaag agccaggctc cgcagtcggc caacaagacc 300
ggcaacgtcg acgacgccaa caaccaggat ccgatgcaag cgctgatgca gctgctggaa 360
gacctggtga agctgctgaa ggcggccctg cacatgcagc agcccggcgg caatgacaag 420
ggcaacggcg tgggcggtgc caacggcgcc aagggtgccg gcggccaggg cggcctggcc 480
gaagcgctgc aggagatcga gcagatcctc gcccagctcg gcggcgggcg tgctggcgcc 540
ggcggcgcgg gtggcggtgt cggcggtgct ggtggcgcgg atggcggctc cggcgcggtg 600
ggcgcgaggc gtgcgaacgg cggcgacggc ggcaatggcg tgaacggcaa ccaggcgaac 660
ggcccgcaga acgcaggcga tgtcaacggt gccaacggcg cggatgacgg cagcgaagac 720
caggggcgcc tcaccggcgt gctgcaaaag ctgatgaaga tcctgaacgc gctggtgcag 780
atgatgcagc aaggcggcct cggcgggcggc aaccaggcgc agggcggtc gaagggtgcc 840
ggcaacgcct cgccggcttc cggcgcgaa cggggcgcg accagcccgg ttcggcggat 900
gatcaatcgt ccggccagaa caatctgcaa tcccagatca tggatgtggt gaaggaggtc 960
gtccagatcc tgcagcagat gctggcgggc cagaacggcg gcagccagca gtccacctcg 1020
acgcagccga tgtaa 1035

The above nucleotide and amino acid sequences are disclosed and further described in U.S. Patent No. 5,776,889 to Wei et al., which is hereby incorporated by reference in its entirety.

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A hypersensitive response elicitor polypeptide or protein derived from *Xanthomonas campestris* has an amino acid sequence corresponding to SEQ. ID.

No. 13 as follows:

5 Met Asp Ser Ile Gly Asn Asn Phe Ser Asn Ile Gly Asn Leu Gln Thr
1 5 10 15
Met Gly Ile Gly Pro Gln Gln His Glu Asp Ser Ser Gln Gln Ser Pro
20 25 30
10 Ser Ala Gly Ser Glu Gln Gln Leu Asp Gln Leu Leu Ala Met Phe Ile
35 40 45
Met Met Met Leu Gln Gln Ser Gln Gly Ser Asp Ala Asn Gln Glu Cys
50 55 60
Gly Asn Glu Gln Pro Gln Asn Gly Gln Gln Glu Gly Leu Ser Pro Leu
65 70 75 80
15 Thr Gln Met Leu Met Gln Ile Val Met Gln Leu Met Gln Asn Gln Gly
85 90 95
Gly Ala Gly Met Gly Gly Gly Gly Ser Val Asn Ser Ser Leu Gly Gly
100 105 110
20 Asn Ala

This hypersensitive response elicitor polypeptide or protein has an estimated molecular weight of about 12 kDa based on the deduced amino acid sequence, which is consistent with a molecular weight of about 14 kDa as detected by SDS-PAGE.

The above protein or polypeptide is encoded by a DNA molecule according to SEQ. ID. No. 14 as follows:

25 atggactcta tcggaaacaa cttttcgaat atcggcaacc tgcagacgat gggcatcggg 60
cctcagcaac acgaggactc cagccagcag tcgccttcgg ctggctccga gcagcagctg 120
gatcagttgc tcgcatgtt catcatgatg atgctgcaac agagccaggg cagcgatgca 180
30 aatcaggagt gtggcaacga acaaccgcag aacgggtcaac aggaaggect gagtccgttg 240
acgcagatgc tgatgcagat cgtgatgcag ctgatgcaga accagggcgg cgccggcatg 300
ggcgggtggcg gttcgggtcaa cagcagcctg ggcgggcaacg cc 342

35 The above nucleotide and amino acid sequences are disclosed and further described in U.S. Patent Application Serial No. 09/829,124, which is hereby incorporated by reference in its entirety.

Other embodiments of the present invention include, but are not limited to, use of a hypersensitive response elicitor protein or polypeptide derived

from *Erwinia carotovora* and *Erwinia stewartii*. Isolation of *Erwinia carotovora* hypersensitive response elicitor protein or polypeptide is described in Cui, et al., "The RsmA Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrp* N_{Ecc} and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI, 9(7):565-73 (1996), which is hereby incorporated by reference in its entirety. A hypersensitive response elicitor protein or polypeptide of *Erwinia stewartii* is set forth in Ahmad, et al., "Harpin is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microbe Interact., July 14-19, 1996 and Ahmad, et al., "Harpin is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am. Phytopath. Soc., July 27-31, 1996, which are hereby incorporated by reference in their entirety.

The hypersensitive response elicitor proteins or polypeptides from various *Phytophthora* species are described in Kaman, et al., "Extracellular Protein Elicitors from Phytophthora: Most Specificity and Induction of Resistance to Bacterial and Fungal Phytopathogens," Molec. Plant-Microbe Interact., 6(1):15-25 (1993); Ricci, et al., "Structure and Activity of Proteins from Pathogenic Fungi Phytophthora Eliciting Necrosis and Acquired Resistance in Tobacco," Eur. J. Biochem., 183:555-63 (1989); Ricci, et al., "Differential Production of Parasiticein, and Elicitor of Necrosis and Resistance in Tobacco, by Isolates of Phytophthora parasitica," Plant Path. 41:298-307 (1992); Baillreul, et al., "A New Elicitor of the Hypersensitive Response in Tobacco: A Fungal Glycoprotein Elicits Cell Death, Expression of Defense Genes, Production of Salicylic Acid, and Induction of Systemic Acquired Resistance," Plant J., 8(4):551-60 (1995), and Bonnet, et al., "Acquired Resistance Triggered by Elicitors in Tobacco and Other Plants," Eur. J. Plant Path., 102:181-92 (1996), which are hereby incorporated by reference in their entirety.

Another hypersensitive response elicitor for use in accordance with the present invention is derived from *Clavibacter michiganensis* subsp. *sepedonicus*. The use of this particular hypersensitive response elicitor is described in U.S. Patent Application Serial No. 09/136,625, which is hereby incorporated by reference in its entirety.

Other elicitors can be readily identified by isolating putative hypersensitive response elicitors and testing them for elicitor activity as described, for example, in Wei, Z-M., et al., "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992), which is hereby incorporated by reference in its entirety. Cell-free preparations from culture supernatants can be tested for elicitor activity (i.e., local necrosis) by using them to infiltrate appropriate plant tissues. Once identified, DNA molecules encoding a hypersensitive response elicitor can be isolated using standard techniques known to those skilled in the art.

The hypersensitive response elicitor protein or polypeptide can also be a fragment of the above hypersensitive response elicitor proteins or polypeptides as well as fragments of full length elicitors from other pathogens.

Suitable fragments can be produced by several means. Subclones of the gene encoding a known elicitor protein can be produced using conventional molecular genetic manipulation for subcloning gene fragments, such as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), and Ausubel et al. (ed.), Current Protocols in Molecular Biology, John Wiley & Sons (New York, NY) (1999 and preceding editions), which are hereby incorporated by reference in their entirety. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller protein or polypeptide that can be tested for elicitor activity, e.g., using procedures set forth in Wei, Z-M., et al., Science 257: 85-88 (1992), which is hereby incorporated by reference in its entirety.

In another approach, based on knowledge of the primary structure of the protein, fragments of the elicitor protein gene may be synthesized using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. Erlich, H.A., et al., "Recent Advances in the Polymerase Chain Reaction," Science 252:1643-51 (1991), which is hereby incorporated by reference in its entirety. These can then be cloned into an appropriate vector for expression of a truncated protein or polypeptide from bacterial cells as described above.

An example of suitable fragments of a hypersensitive response elicitor which elicit a hypersensitive response are fragments of the *Erwinia amylovora*

hypersensitive response elicitor protein or polypeptide of SEQ. ID. No. 3. The fragments can be a C-terminal fragment of the amino acid sequence of SEQ. ID. No. 3, an N-terminal fragment of the amino acid sequence of SEQ. ID. No. 3, or an internal fragment of the amino acid sequence of SEQ. ID. No. 3. The C-terminal
5 fragment of the amino acid sequence of SEQ. ID. No. 3 can span amino acids 105 and 403 of SEQ. ID. No. 3. The N-terminal fragment of the amino acid sequence of SEQ. ID. No. 3 can span the following amino acids of SEQ. ID. No. 3: 1 and 98, 1 and 104, 1 and 122, 1 and 168, 1 and 218, 1 and 266, 1 and 342, 1 and 321, and 1 and 372. The
10 internal fragment of the amino acid sequence of SEQ. ID. No. 3 can span the following amino acids of SEQ. ID. No. 3: 76 and 209, 105 and 209, 99 and 209, 137 and 204, 137 and 200, 109 and 204, 109 and 200, 137 and 180, and 105 and 180. DNA molecules encoding these fragments can also be utilized in the chimeric gene of the present invention.

DNA molecules encoding a hypersensitive response elicitor protein or
15 polypeptide can also include a DNA molecule that hybridizes under stringent conditions to the DNA molecule having nucleotide sequence of SEQ. ID. Nos. 2, 4, 6, 8, 10, 12, or 14. An example of suitable stringency conditions is when hybridization is carried out at a temperature of about 37°C using a hybridization medium that includes 0.9M sodium citrate ("SSC") buffer, followed by washing with 0.2x SSC
20 buffer at 37°C. Higher stringency can readily be attained by increasing the temperature for either hybridization or washing conditions or increasing the sodium concentration of the hybridization or wash medium. Nonspecific binding may also be controlled using any one of a number of known techniques such as, for example, blocking the membrane with protein-containing solutions, addition of heterologous
25 RNA, DNA, and SDS to the hybridization buffer, and treatment with RNase. Wash conditions are typically performed at or below stringency. Exemplary high stringency conditions include carrying out hybridization at a temperature of about 42°C to about 65°C for up to about 20 hours in a hybridization medium containing 1M NaCl, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 0.2%
30 ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, and 50 µg/ml *E. coli* DNA, followed by washing carried out at between about 42°C to about 65°C in a 0.2x SSC buffer.

Variants of suitable hypersensitive response elicitor proteins or polypeptides can also be expressed. Variants may be made by, for example, the deletion, addition, or alteration of amino acids that have minimal influence on the properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

When it is desirable to perform the methods of the present invention with application of the hypersensitive response elicitor protein or polypeptide to a plant seed or a plant, it is preferable, though not necessary, that the hypersensitive response elicitor protein or polypeptide be applied in isolated form or with a carrier as discussed hereinafter.

One particular hypersensitive response elicitor protein, known as harpin_{Ea}, is commercially available from Eden Bioscience Corporation (Bothell, Washington) under the name of Messenger[®]. Messenger[®] contains 3% by weight of harpin_{Ea} as the active ingredient and 97% by weight inert ingredients. Harpin_{Ea} is one type of hypersensitive response elicitor protein from *Erwinia amylovora*, identified herein by SEQ. ID. No. 3.

Alternatively, the hypersensitive response elicitor protein or polypeptide can be recombinantly produced, isolated, and then purified, if necessary. When recombinantly produced, the hypersensitive response elicitor protein or polypeptide is expressed in a recombinant host cell, typically, although not exclusively, a prokaryote.

When a prokaryotic host cell is selected for subsequent transformation, the promoter region used to construct the recombinant DNA molecule (i.e., transgene) should be appropriate for the particular host. The DNA sequences of eukaryotic promoters, as described *infra* for plants, differ from those of prokaryotic promoters. Eukaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a prokaryotic system, and, further, prokaryotic promoters are not recognized and do not function in eukaryotic cells.

Similarly, translation of mRNA in prokaryotes depends upon the presence of the proper prokaryotic signals which differ from those of eukaryotes. Efficient translation of mRNA in prokaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference in its entirety.

Promoters vary in their "strength" (i.e., their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, *lac* promoter, *trp* promoter, *recA* promoter, ribosomal RNA promoter, the P_R and P_L promoters of coliphage lambda and others, including but not limited, to *lacUV5*, *ompF*, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lacUV5 (tac)* promoter or other *E. coli* promoters produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promoter unless specifically induced. In certain operons, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in prokaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific

messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promoter, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires a Shine-Dalgarno ("SD") sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include, but are not limited to, the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the DNA molecule coding for a hypersensitive response elicitor protein or polypeptide has been ligated to its appropriate regulatory regions using well known molecular cloning techniques, it can then be introduced into a vector or otherwise introduced directly into a host cell (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press, NY (1989), which is hereby incorporated by reference in its entirety).

The recombinant molecule can be introduced into host cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like. Preferably the host cells are either a bacterial cell or a plant cell. The host cells, when grown in an appropriate medium, are capable of expressing the hypersensitive response elicitor protein or polypeptide, which can then be isolated therefrom and, if necessary, purified.

The hypersensitive response elicitor protein or polypeptide of the present invention is preferably produced in purified form (preferably at least about 60%, more preferably 80%, pure) by conventional techniques. Typically, the protein or polypeptide of the present invention is produced but not secreted into the growth medium of recombinant host cells, usually although not exclusively bacterial host cells. Alternatively, the protein or polypeptide of the present invention is secreted into growth medium.

In the case of an unsecreted hypersensitive response elicitor protein or polypeptide, the protein or polypeptide can be isolated from the host cell (e.g., *E. coli*)

carrying a recombinant plasmid by lysing the host cell with sonication, heat, or chemical treatment, after which the homogenate is centrifuged to remove bacterial debris. The supernatant is then subjected to heat treatment and the hypersensitive response elicitor is separated by centrifugation. The supernatant fraction containing the hypersensitive response elicitor protein is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction may be further purified by ion exchange or HPLC.

Alternatively, it is desirable for recombinant host cells to secrete the hypersensitive response elicitor protein or polypeptide into growth medium, thereby avoiding the need to lyse cells and remove cellular debris. To enable the host cell to secrete the hypersensitive response elicitor, the host cell can also be transformed with a type III secretion system in accordance with Ham et al., "A Cloned *Erwinia chrysanthemi* Hrp (Type III Protein Secretion) System Functions in *Escherichia coli* to Deliver *Pseudomonas syringae* Avr Signals to Plant Cells and Secrete Avr Proteins in Culture," Microbiol. 95:10206-10211 (1998), which is hereby incorporated by reference in its entirety. After growing recombinant host cells which secrete the hypersensitive response elicitor into growth medium, isolation of the hypersensitive response elicitor protein or polypeptide from growth medium can be carried out substantially as described above.

The methods of the present invention which involve application of the hypersensitive response elicitor polypeptide or protein can be carried out through a variety of procedures when all or part of the plant is treated, including leaves, stems, roots, etc. This may (but need not) involve infiltration of the hypersensitive response elicitor polypeptide or protein into the plant. Suitable application methods include high or low pressure spraying, injection, dusting, and leaf abrasion proximate to when elicitor application takes place. More than one application of the hypersensitive response elicitor protein or polypeptide may be desirable either to realize maximal benefit of the value-added trait or overcome a yield penalty, particularly over the course of a growing season. When treating plant seeds in accordance with the application embodiment of the present invention, the hypersensitive response elicitor protein or polypeptide can be applied by low or high pressure spraying, coating, immersion, dusting, or injection. Other suitable application procedures can be

envisioned by those skilled in the art provided they are able to effect contact of the hypersensitive response elicitor polypeptide or protein with cells of the plant or plant seed. Once treated with the hypersensitive response elicitor of the present invention, the seeds can be planted in natural or artificial soil and cultivated using conventional procedures to produce plants. After plants have been propagated from seeds treated in accordance with the present invention, the plants may also be treated with one or more applications of the hypersensitive response elicitor protein or polypeptide. Such propagated plants may, in turn, be useful in producing seeds or propagules (e.g., cuttings) that produce plants capable of insect control.

The hypersensitive response elicitor polypeptide or protein can be applied to plants or plant seeds in accordance with the present invention alone or in a mixture with other materials. Alternatively, the hypersensitive response elicitor polypeptide or protein can be applied separately to plants with other materials being applied at different times.

A composition suitable for treating plants or plant seeds in accordance with the application embodiment of the present invention contains a hypersensitive response elicitor polypeptide or protein in a carrier. Suitable carriers include water, aqueous solutions, slurries, or dry powders. In this embodiment, the composition contains greater than 500 nM hypersensitive response elicitor polypeptide or protein.

Although not required, this composition may contain additional additives including fertilizer, insecticide, fungicide, nematocide, herbicide, and mixtures thereof. Suitable fertilizers include $(\text{NH}_4)_2\text{NO}_3$. An example of a suitable insecticide is Malathion. Useful fungicides include Captan.

Other suitable additives include buffering agents, wetting agents, coating agents, and abrading agents. These materials can be used to facilitate the process of the present invention. In addition, the hypersensitive response elicitor polypeptide or protein can be applied to plant seeds with other conventional seed formulation and treatment materials, including clays and polysaccharides.

Although application of the hypersensitive response elicitor protein or polypeptide is preferably carried out in isolated form or with a carrier, the hypersensitive response elicitor protein or polypeptide can also be applied in a non-isolated but non-infectious form. When applied in non-isolated but non-infectious

form, the hypersensitive response elicitor is applied indirectly to the plant via application of a bacteria which expresses and then secretes or injects the expressed hypersensitive response elicitor protein or polypeptide into plant cells or tissues. Such application can be carried out by applying the bacteria to all or part of a plant or a plant seed under conditions where the polypeptide or protein contacts all or part of the cells of the plant or plant seed. Alternatively, the hypersensitive response elicitor protein or polypeptide can be applied to plants such that seeds recovered from such plants themselves are able to enhance plant growth, impart stress tolerance in plants, impart disease resistance in plants, and/or to effect insect control.

The embodiment of the present invention where the hypersensitive response elicitor polypeptide or protein is applied to the plant or plant seed in a non-isolated but non-infectious form can be carried out in a number of ways, including: 1) application of bacteria which do not cause disease and are transformed with genes encoding a hypersensitive response elicitor polypeptide or protein, and 2) application of bacteria which cause disease in some plant species (but not in those to which they are applied) and naturally contain a gene encoding the hypersensitive response elicitor polypeptide or protein.

In one embodiment of the bacterial application mode of the present invention, the bacteria do not cause disease and have been transformed (e.g., recombinantly) with genes encoding a hypersensitive response elicitor polypeptide or protein. For example, *E. coli*, which does not elicit a hypersensitive response in plants, can be transformed with genes encoding a hypersensitive response elicitor polypeptide or protein and then applied to plants. Bacterial species other than *E. coli* can also be used in this embodiment of the present invention.

In another embodiment of the bacterial application mode of the present invention, the bacteria do cause disease and naturally contain a gene encoding a hypersensitive response elicitor polypeptide or protein. Examples of such bacteria are noted above. However, in this embodiment, these bacteria are applied to plants or their seeds which are not susceptible to the disease carried by the bacteria. For example, *Erwinia amylovora* causes disease in apple or pear but not in tomato. However, such bacteria will elicit a hypersensitive response in tomato. Accordingly, in accordance with this embodiment of the present invention, *Erwinia amylovora* can

be applied to tomato plants or seeds to enhance growth without causing disease in that species.

Another aspect of the present invention is a method which is carried out by providing a plant cell, transforming the plant cell with (i) a first DNA molecule encoding a transcript or a protein or polypeptide which confers a trait to a plant grown from the transformed plant cell and (ii) a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide which is different than the protein or polypeptide encoded by the first DNA molecule, the transforming being carried out under conditions effective to produce a transformed plant cell, and then regenerating a transgenic plant from the transformed plant cell. By transforming the plant cell with the second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide, as discussed *infra*, the resulting transgenic plant expresses the hypersensitive response elicitor and exhibits enhanced growth, stress tolerance, disease resistance, or insect resistance.

According to one embodiment, transforming with the second DNA molecule imparts enhanced growth, stress tolerance, disease resistance, or insect resistance to the plant, thereby maximizing benefit to the plant of the trait conferred by transforming with the first DNA molecule. For example, when the particular trait conferred by the first DNA molecule relates to specific but limited growth enhancement, stress tolerance, disease resistance, or insect resistance of a transgenic plant, this embodiment relates to conferring broad growth enhancement, stress tolerance, disease resistance, or insect resistance that complements the specific but limited trait.

According to another embodiment, transforming with the first DNA molecule is accompanied by a deleterious effect on growth, stress tolerance, disease resistance, or insect resistance, and transforming with the second DNA molecule overcomes the deleterious effect. Thus, this aspect of the present invention is also directed to overcoming a yield penalty resulting from a trait.

Any of the above-described DNA molecules encoding a hypersensitive response elicitor protein or polypeptide can be used to prepare a desired transgenic plant that expresses both a transgene conferring a value-added trait and a transgene encoding a hypersensitive response elicitor.

The transgene or DNA molecule conferring a trait can be any DNA molecule that confers a value-added trait to a transgenic plant. The value-added trait can be for disease resistance, insect resistance, enhanced growth, herbicide resistance, stress tolerance, male sterility, modified flower color, or biochemically modified plant product. Biochemically modified plant products can include, without limitation, modified cellulose in cotton, modified ripening of fruits or vegetables, modified flavor of fruits or vegetables, modified flower color, expression of industrial enzymes, modified starch content, modified dietary fiber content, modified sugar metabolism, modified food quality or nutrient content, and bioremediation.

The transgene or DNA molecule conferring a value-added trait can encode either a transcript (sense or antisense) or a protein or polypeptide which is different from the hypersensitive response elicitor protein or polypeptide. Either the transcript or the protein or polypeptide, or both, can confer the value-added trait.

A number of proteins or polypeptides which can confer a value-added trait are known in the art and others are continually being identified, isolated, and expressed in host plants. Suitable proteins or polypeptides which can be encoded by the transgene or DNA molecule conferring a value-added trait include, without limitation, B.t. toxin, *Photorhabdus luminescens* protein, protease inhibitors, amylase inhibitors, lectins, chitinases, endochitinase, chitobiase, defensins, osmotins, crystal proteins, virus proteins, herbicide resistance proteins, mannitol dehydrogenase, PG inhibitors, ACC degradation proteins, barnase, phytase, fructans, invertase, and SAMase.

A number of transcripts which can confer a value-added trait are known in the art and others are continually being identified, isolated, and expressed in host plants. The transcript encoded by the transgene or DNA molecule conferring a trait can be either a sense RNA molecule, which is translatable or untranslatable, or an antisense RNA molecule capable of hybridizing to a target RNA or protein. Suitable transcripts which can be encoded by the transgene or DNA molecule conferring a trait include, without limitation, translatable and untranslatable RNA transcripts capable of interfering with plant virus pathogenesis (de Haan et al., "Characterization of RNA-Mediated Resistance to Tomato Spotted Wilt Virus in Transgenic Tobacco Plants," BioTechnology 10:1133-1137 (1992); Pang et al., "Nontarget DNA

Sequences Reduce the Transgene Length Necessary for RNA-Mediated Tospovirus Resistance in Transgenic Plants,” Proc. Natl. Acad. Sci. USA 94:8261-8266 (1997), which are hereby incorporated by reference in their entirety) and antisense RNA molecules which interfere with the activity of an enzyme (e.g., starch synthase, ACC oxidase, pectinmethylesterase, polygalacturonase, etc.) or the synthesis of a particular product (e.g., glycoalkaloid synthesis).

Exemplary expression products of the transgene or DNA molecule conferring a trait and their uses are identified in Table 1 below.

Table 1: Expression Products of Transgene Conferring Value-Added Trait and Their Uses

Trait and Expression Product	Reference
<u>Pest/Pathogen Resistance</u>	
B.t. toxin	U.S. Patent No. 5,990,383 to Warren et al.
crystal proteins	U.S. Patent No. 4,996,155 to Sick et al.
<i>Photorhabdus luminescens</i> protein	Bowen et al., <u>Science</u> 280:2129 (1998)
protease inhibitors	Ryan, <u>Annu. Rev. Phytopathol.</u> 38:425-449 (1990)
amylase inhibitors	Mundy et al., <u>Planta</u> 169:51-63 (1986)
lectins	EP Patent Application No. 351,924 A to Shell
chitinase (nematode & fungal)	U.S. Patent No. 5,290,687 to Suslow et al.
endochitinase & chitobiase	U.S. Patent No. 5,378,821 to Harman et al.
endochitinase activity	U.S. Patent No. 5,446,138 to Blaiseu et al.
defensins	U.S. Patent No. 4,705,777 to Lehrer et al.
osmotins	Liu et al., <u>PNAS USA</u> 91:1888 (1994)
tobacco mosaic virus coat protein	Beachy et al., <u>Rev. Phytopathol.</u> 28:451-474 (1990)
cucumber mosaic virus coat protein	U.S. Patent No. 5,349,128 to Quemada et al.
potato coat protein	U.S. Patent No. 4,970,168 to Tumer et al.
potato leaf roll virus coat protein	U.S. Patent No. 5,304,730 to Lawson et al.
potato virus replicase	U.S. Patent No. 5,503,999 to Jilka et al.
	U.S. Patent No. 5,510,253 to Mitsky et al.
potyvirus coat protein	WO 90/02184 to Gonsalves et al.
<u>Herbicide Resistance</u>	
glyphosate resistance (EPSP synthase protein)	U.S. Patent No. 4,535,060 to Comai et al.
chlorsulfuron resistance	Haughn et al., <u>Mol. Gen. Genet.</u> 211:266 (1988)
phosphinothriun/bialaphos resistance	De Block, <u>EMBO J.</u> 6:2513 (1987)
<u>Improved Nutrient Content</u>	
protein	U.S. Patent No. 6,057,493 to Willmitzer et al.
vitamins	U.S. Patent No. 5,750,872 to Bennett et al.
oils	Shintani et al., <u>Plant Physiol.</u> 114(3):881-886 (1997); U.S. Patent No. 6,069,298 to Gengenbach et al.

Table 1 cont.

Trait and Expression Product	Reference
<u>Stress Tolerance</u>	
cold	U.S. Patent No. 5,891,859 to Thomashow et al.
metals	U.S. Patent No. 5,668,294 to Meaghar et al.
drought	U.S. Patent No. 5,563,324 to Tarczynski et al.
	U.S. Patent No. 5,780,709 to Adams et al.
<u>Secondary Compounds</u>	
PHB	Poirier et al., <u>Science</u> 256:520 (1992); Poirier et al., <u>Bio/Technology</u> 13:142 (1995) Tavladorki et al., <u>Nature</u> 366:469 (1993) EP Patent Application No. 436,003 A to Sijmons et al.
antibodies	
pharmaceutical peptides	
<u>Improved Fiber</u>	
cotton	U.S. Patent No. 5,932,713 to Kasukabe et al.
<u>Modified Ripening</u>	
PG inhibition	U.S. Patent No. 5,942,657 to Bird et al.
block ethylene synthesis: ACC	U.S. Patent No. 5,723,766 to Theologis et al.;
degradation	U.S. Patent No. 5,886,164 to Bird et al.
S-adenosylmethionine hydrolase	U.S. Patent No. 5,723,746 to Bestwick et al.
<u>Male Sterility</u>	
barnase	Hartley, <u>J. Mol. Biol.</u> 202:913 (1988)
(Bacillus amyloliquefaciens)	
ribonucleases	EP Patent No. 344,029 to Mariani et al.
(RNase T1 from Aspergillus oryzae)	
<u>Industrial Enzymes</u>	
phytase	U.S. Patent No. 5,593,963 to Van Ooijen et al.; Van Hartingsveldt et al., <u>Gene</u> 127:87 (1993)
<u>Flower Color</u>	
pH gene products	U.S. Patent No. 5,534,660 to Chuck et al. U.S. Patent No. 5,910,627 to Chuck et al. U.S. Patent No. 5,410,096 to Meyer et al. U.S. Patent No. 5,034,323 to Jorgensen et al.
dihydroflavonol 4-reductase	
flavonoid biosynthetic pathway gene	
<u>Starch Content</u>	
anti-sense starch synthase	U.S. Patent No. 6,057,493 to Willmitzer et al.
amylose content	U.S. Patent No. 6,066,782 to Kossman et al.
<u>Dietary Fiber</u>	
potato increased fructans	U.S. Patent No. 5,986,173 to Smeekens et al.
<u>Improved Flavor</u>	
alcohol dehydrogenase II	U.S. Patent No. 6,011,199 to Speirs et al.
pH gene products	U.S. Patent No. 5,534,660 to Chuck et al.
	U.S. Patent No. 5,910,627 to Chuck et al.
sweetness (monellin/thaumatococcus)	U.S. Patent No. 5,739,409 to Fischer et al.

Table 1 cont.

Trait and Expression Product	Reference
<u>Bioremediation</u>	
metalothionein in <i>Brassicaceae</i>	U.S. Patent No. 5,364,451 to Raskin et al.
<u>Modified Sugar Metabolism</u>	
invertase	U.S. Patent No. 5,917,127 to Willmitzer et al.
<u>Modified Food Quality</u>	
altered carbohydrate composition	WO 90/12876 to Gausing et al.
increased glutenin (wheat & others)	U.S. Patent No. 5,914,450 to Blechl et al.
increased storage lipids in seed	U.S. Patent No. 5,914,449 to Murase et al.

Each of the references listed in Table 1 is hereby incorporated by reference in its entirety.

To express, in plant tissues, the DNA molecule encoding a hypersensitive response elicitor protein or polypeptide and/or the DNA molecule conferring a value-added trait, the coding regions must be ligated to appropriate regulatory regions which are operable in plant tissues. Therefore, plant expressible promoters and 3' polyadenylation regions must be ligated to the DNA molecules to afford a transgene which can then be used to transform plant cells or tissues.

Any plant-expressible promoter can be utilized regardless of its origin, i.e., viral, bacterial, plant, etc. Without limitation, two suitable promoters include the nopaline synthase promoter (Fraley et al., "Expression of Bacterial Genes in Plant Cells," Proc. Natl. Acad. Sci. USA 80:4803-4807 (1983), which is hereby incorporated by reference in its entirety) and the cauliflower mosaic virus 35S promoter (O'Dell et al., "Identification of DNA Sequences Required for Activity of the Cauliflower Mosaic Virus 35S Promoter," Nature, 313(6005):810-812 (1985), which is hereby incorporated by reference in its entirety). Both of these promoters yield constitutive expression of coding sequences under their regulatory control.

While constitutive expression is generally suitable for expression of transgenes, it should be apparent to those of skill in the art that temporally or tissue regulated expression may also be desirable, in which case any regulated promoter can be selected to achieve the desired expression. Typically, the temporally or tissue regulated promoters will be used in connection with DNA molecules that are expressed at only certain stages of development or only in certain tissues.

For example, the E4 and E8 promoters of tomato have been used to direct fruit-specific expression of a DNA sequence in transgenic tomato plants

(Cordes et al., Plant Cell 1:1025-1034 (1989); Deikman et al., EMBO J. 7:3315-3320 (1988); and Della Penna et al., Proc. Natl. Acad. Sci. USA 83:6420-6424 (1986), which are hereby incorporated by reference in their entirety). Another fruit-specific promoter is the PG promoter (Bird et al., Plant Molec. Biol. 11:651-662 (1988), which is hereby incorporated by reference). Another tissue-specific promoter is the AP2 promoter from the ovule-specific BEL1 gene promoter described in Reiser et al., Cell 83:735-742 (1995), which is hereby incorporated by reference in its entirety.

Promoters useful for expression in seed tissues include, without limitation, the promoters from genes encoding seed storage proteins, such as napin, cruciferin, phaseolin, and the like (see U.S. Patent No. 5,420,034 to Kridl et al., which is hereby incorporated by reference in its entirety). Other suitable promoters include those from genes encoding embryonic storage proteins.

Promoters useful for expression in leaf tissue include the Rubisco small subunit promoter.

Promoters useful for expression in tubers, particularly potato tubers, include the patatin promoter.

In another embodiment of the present invention, expression of one or both transgenes is environmentally-regulated, i.e., through the use of an inducible promoter. Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, elevated temperature, or the presence of light. In some plants, it may also be desirable to use promoters which are responsive to pathogen infiltration or stress. For example, it may be desirable to limit expression of the hypersensitive response elicitor protein or polypeptide in response to infection by a particular pathogen of the plant. One example of a pathogen-inducible promoter is the *gst1* promoter from potato, which is described in U.S. Patent Nos. 5,750,874 and 5,723,760 to Strittmayer et al., which are hereby incorporated by reference in their entirety.

Expression of the transgenes in isolated plant cells or tissue or whole plants also requires appropriate transcription termination and polyadenylation of mRNA. Any 3' regulatory region suitable for use in plant cells or tissue can be operably linked to the coding regions in the transgenes. A number of 3' regulatory regions are known to be operable in plants. Exemplary 3' regulatory regions include,

without limitation, the nopaline synthase 3' regulatory region (Fraley, et al.,
"Expression of Bacterial Genes in Plant Cells," Proc. Nat'l. Acad. Sci. USA, 80:4803-
4807 (1983), which is hereby incorporated by reference in its entirety) and the
cauliflower mosaic virus 3' regulatory region (Odell, et al., "Identification of DNA
Sequences Required for Activity of the Cauliflower Mosaic Virus 35S Promoter,"
Nature, 313(6005):810-812 (1985), which is hereby incorporated by reference in its
entirety).

The promoter and a 3' regulatory region can readily be ligated to DNA
molecules using well known molecular cloning techniques described in Sambrook et
al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor
Press, NY (1989), which is hereby incorporated by reference in its entirety.

In some instances, it may be desirable for the hypersensitive response
elicitor to be secreted by the cells in which it is expressed into intercellular regions of
the plant. Thus, it may be desirable to ligate a DNA molecule encoding a secretion
signal to the coding region of the transgene coding for the hypersensitive response
elicitor protein or polypeptide. A number of suitable secretion signals are known in
the art and others are continually being identified. The secretion signal can be an
RNA leader which directs secretion of the subsequently transcribed protein or
polypeptide, or the secretion signal can be an amino terminal peptide sequence that is
recognized by a host plant secretory pathway. Typically, the DNA molecule encoding
the secretion signal can be ligated between the promoter and the coding region using
known molecular cloning techniques as indicated above.

An exemplary secretion signal is the secretion signal polypeptide for
PR1-b gene of *Nicotiana tabacum*. The DNA molecule encoding this secretion signal
has a nucleotide sequence corresponding to SEQ. ID. No. 15 as follows:

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cacgaagctt accatgggat tttttctctt ttcacaaatg ccttcatttt ttcttggtgc 60
gacattcttc ttattcctaa taatatctca ctcttctcat gcccaaaact cccgcggrga 120
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The polypeptide encoded by this nucleic acid molecule has an amino acid sequence
corresponding to SEQ. ID. No. 16 as follows:

Met	Gly	Phe	Phe	Leu	Phe	Ser	Gln	Met	Pro	Ser	Phe	Phe	Leu	Val	Ser
1				5				10					15		
Thr	Leu	Leu	Leu	Phe	Leu	Ile	Ile	Ser	His	Ser	Ser	His	Ala	Gln	Asn
			20					25					30		
5	Ser	Arg	Gly												
			35												

Once transgenes of the type described above have been prepared, they can be introduced into plant cells or tissues for subsequent regeneration of whole plants. Thus, another aspect of the present invention relates to a transgenic plant which has been treated or genetically modified so that the transgenic plant can either exhibit enhanced growth, disease resistance, stress resistance, or insect resistance to realize the maximum benefit of a value-added trait or otherwise overcome a yield penalty concomitant with a value-added trait.

According to a one embodiment, the transgenic plant of the present invention includes a DNA molecule encoding a transcript or a protein or polypeptide that confers a trait, wherein the transgenic plant or a plant seed from which the transgenic plant is grown, is treated with a hypersensitive response elicitor protein or polypeptide under conditions effective to impart enhanced growth, disease resistance, stress resistance, or insect resistance to the transgenic plant.

According to another embodiment, the transgenic plant of the present invention including a first DNA molecule encoding a transcript or a protein or polypeptide that confers a trait and a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide different than the protein or polypeptide encoded by the first DNA molecule. Because the transgenic plant includes at least two DNA molecules, the first and second DNA molecules can be inserted into a plant cell or tissue either individually (i.e., in separate constructs used during separate transformation steps) or simultaneously (i.e., in a single construct or in separate constructs used during a single transformation step).

Another aspect of the present invention relates to a system for use in transforming plants with multiple DNA molecules, typically although not exclusively during separate transformation events. This system includes a first DNA construct that includes a DNA molecule encoding a transcript or a protein or polypeptide which confers a trait to a host plant, and a second DNA construct that contains a DNA

molecule encoding a hypersensitive response elicitor protein or polypeptide which is different from the protein or polypeptide encoded by the DNA molecule of the first DNA construct. The first and second DNA molecules can be of the type described above. The first and second DNA constructs each contain a promoter operably linked 5' to the DNA molecule (e.g., first or second DNA molecule) and a 3' regulatory region operably linked to the DNA molecule.

A further aspect of the present invention relates to a DNA construct for use in transforming plants with multiple DNA molecules, typically during a single transformation event. The DNA construct includes a first DNA molecule encoding a transcript or a protein or polypeptide which confers a value-added trait to a host plant and a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide which is different from any protein or polypeptide encoded by the first DNA molecule. The first and second DNA molecules can be of the type described above. The DNA construct can include a first promoter operable in plant cells operably linked 5' to one or both of the first and second DNA molecules.

Alternatively, where the first promoter is only operably linked to the first DNA molecule, the DNA construct can also include a second promoter operably coupled to the second DNA molecule. The first and second promoters can be the same or different. Generally, both the first and second DNA molecules will be ligated to a 3' regulatory region, which can be the same or different for each of the first and second DNA molecules.

Both the transgene or DNA molecule conferring a value-added trait and the transgene or DNA molecule encoding the hypersensitive response elicitor protein or polypeptide can be incorporated into cells using conventional recombinant DNA technology. Generally, this involves inserting the transgenes or DNA molecules into expression vector(s) or system(s) to which they are heterologous (i.e., not normally present). Because either single or multiple expression systems can be used, a single expression system can include a vector into which is inserted both the first DNA construct containing the first DNA molecule and the second DNA construct containing the second DNA molecule. Alternatively, the expression system can include two vectors into which are inserted one or the other of the first DNA construct containing the first DNA molecule and the second DNA construct containing the

second DNA molecule. The first and second DNA molecules can be ligated to the appropriate promoter(s) and 3' regulatory regions either before insertion into the expression vector(s) or system(s) or at the time of their insertion therein.

U.S. Patent No. 4,237,224 issued to Cohen and Boyer, which is hereby incorporated by reference in its entirety, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including prokaryotic organisms, typically bacteria, and eukaryotic cells grown in tissue culture, typically plant cells.

As indicated above, several aspects of the present invention are directed to the preparation of transgenic plants. Basically, this is carried out by providing a plant cell (which may or may not already possesses a transgene), transforming the plant cell with one or more transgenes of the type described above under conditions effective to yield expression of such transgenes, and then regenerating the transformed cells into whole transgenic plants. Preferably the transgene(s) is stably inserted into the genome of the transformed plant cell and whole plants regenerated therefrom.

One approach to transforming plant cells with the transgenes or DNA molecules identified herein is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford, et al., which are hereby incorporated by reference in their entirety. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector(s) containing the DNA to be used in transforming the plant cell. Alternatively, the target cell can be surrounded by the vector(s) so that the vector(s) is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the

vector and DNA) can also be propelled into plant cells. Other variations of particle bombardment, now known or hereafter developed, can also be used.

Another method of introducing the transgenes or DNA molecules identified herein is fusion of protoplasts with other entities, either minicells, cells, lysosomes, or other fusible lipid-surfaced bodies that contain the first and second transgenes or DNA molecules. Fraley et al., Proc. Natl. Acad. Sci. USA, 79:1859-63 (1982), which is hereby incorporated by reference in its entirety.

The transgenes or DNA molecules identified herein may also be introduced into the plant cells by electroporation. Fromm, et al., Proc. Natl. Acad. Sci. USA, 82:5824 (1985), which is hereby incorporated by reference in its entirety. In this technique, plant protoplasts are electroporated in the presence of plasmids containing the transgenes or DNA molecules. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

Another method of introducing the transgenes or DNA molecules identified herein into plant cells is to infect a plant cell with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* previously transformed with one or both of the transgenes or DNA molecules identified herein. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Agrobacterium is a representative genus of the Gram-negative family Rhizobiaceae. Its species are responsible for crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

The transgenes or DNA molecules identified herein can be introduced into appropriate plant cells by means of the Ti plasmid of *A. tumefaciens* or the Ri

plasmid of *A. rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells upon infection by *Agrobacterium* and is stably integrated into the plant genome. Schell, J., Science, 237:1176-83 (1987), which is hereby incorporated by reference in its entirety.

5 Plant tissue suitable for transformation include leaf tissue, root tissue, meristems, zygotic and somatic embryos, and anthers.

After transformation, the transformed plant cells can be selected and regenerated.

10 Preferably, transformed cells are first identified using, e.g., a selection marker simultaneously introduced into the host cells along with the transgene or DNA molecules identified herein. Suitable selection markers include, without limitation, markers coding for antibiotic resistance, such as kanamycin resistance (Fraley, et al., Proc. Natl. Acad. Sci. USA, 80:4803-4807 (1983), which is hereby incorporated by reference in its entirety). A number of antibiotic-resistance markers are known in the art and other are continually being identified. Any known antibiotic-resistance
15 marker can be used to transform and select transformed host cells in accordance with the present invention. Cells or tissues are grown on a selection media containing an antibiotic, whereby generally only those transformants expressing the antibiotic resistance marker continue to grow.

20 Once a recombinant plant cell or tissue has been obtained, it is possible to regenerate a transgenic plant of the present invention. Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and
25 Vol. III (1986), which are hereby incorporated by reference in their entirety.

30 It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major crop and medicinal plant species, trees, perennial and annual ornamental plants, and turf and ornamental grasses. Exemplary crop species include, without limitation, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, canola, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear,

melon, strawberry, cranberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane. Exemplary trees include, without limitation, maple, birch, oak, walnut, cherry, pine, and poplar. Exemplary ornamental plants include, without limitation, begonias, impatiens, geraniums, lilies, daylilies, irises, tulips, and roses.

5 Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form
10 plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and
15 repeatable.

 After the transgenes or DNA molecules identified herein are stably incorporated in transgenic plants, they can be transferred to other plants by sexual crossing or by preparing cultivars. With respect to sexual crossing, any of a number of standard breeding techniques can be used depending upon the species to be
20 crossed. Cultivars can be propagated in accord with common agricultural procedures known to those in the field. Once transgenic plants of this type are produced, the plants themselves can be cultivated in accordance with conventional procedures. Alternatively, transgenic seeds or propagules (e.g., scion or rootstock cultivars) are recovered from the transgenic plants.

25 A further aspect of the present invention relates to a method of making a transgenic plant which includes providing a transgenic plant seed containing both the transgene or DNA molecule conferring the trait and the transgene or DNA molecule encoding the hypersensitive response elicitor protein or polypeptide, and then planting the transgenic seed under conditions effective to grow a transgenic plant
30 from the transgenic seed. Although any medium can be used to germinate and grow the transgenic seeds, preferably they are planted in the soil and cultivated using conventional procedures to produce the transgenic plants. Preferably, the transgenic

plant seed is harvested from a transgenic parent plant as described above. Thus, the transgenic plants are propagated from the planted transgenic seeds under conditions effective to confer the value-added trait and hypersensitive response elicitor protein or polypeptide expression to subsequent generations.

Another method for preparing a transgenic plant of the present invention involves providing two distinct transgenic plant lines, one containing the transgene or DNA molecule conferring the trait stably inserted into its genome and the other containing the transgene or DNA molecule encoding the hypersensitive response elicitor protein or polypeptide stably inserted into its genome. The two lines are then crossed using conventional breeding techniques and the resulting generation segregated and self-crossed to propagate a single hybrid line which possesses the value-added trait conferred by expression of the first transgene or DNA molecule and expresses the hypersensitive response elicitor protein or polypeptide encoded by the second transgene or DNA molecule. Additional value-added traits can be crossed into such a transgenic hybrid line.

EXAMPLES

The following examples are provided to illustrate embodiments of the present invention, but they are by no means intended to limit its scope.

Example 1 - Increased Yields From Transgenic Cotton Varieties Treated With Messenger

Field trials designed to test the effects of Messenger[®] on disease resistance and crop yield in cotton were performed using the following transgenic cotton varieties: Delta and Pine Land ("DPL") 20B, DPL 33B, DPL 35B, DPL 50B, Stoneville BXN 47, and Paymaster 1220BR. All of the DPL cotton varieties are transgenic for genes encoding Bt toxin, which confers resistance to a specific class of insects. Stoneville BXN 47 is a transgenic cotton variety with a gene for resistance to the herbicide bromoxynil. Paymaster 1220BR is a transgenic variety with stacked transgenic traits: in addition to the Bt toxin gene, this variety carries a second gene conferring resistance to the herbicide glyphosate. The transgenic traits in all six

varieties have specific functions limited to providing insect resistance and/or resistance to herbicide. The transgenes are not intended to alter capacity for growth and yield so the characteristics of the non-transgenic parental varieties are retained in the transgenic varieties.

5 In the eight field trials where the cotton varieties were transgenic, Messenger[®] was applied by foliar spray or combined seed treatment and foliar spray. In five of these trials, different numbers of treatments and rates of application were tested. Yields were measured in lbs lint/acre or in lbs seed cotton/acre. The results of the trials are summarized in Table 2 below.

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Table 2: Increased Yields From Messenger[®] Treated Transgenic Cotton

Trial	Cotton Variety	Treatment	Rate	Percent Yield Increase
1	DPL 20B	4 foliar	2.2 oz./acre	11.4
2	DPL 33B	3 foliar	2.2 oz./acre	11.4
3	DPL 33B	3 foliar	2.2 oz./acre	5.9
		3 foliar	4.4 oz./acre	9.5
4	DPL 50B	3 foliar	2.29 oz./acre	1.0
		3 foliar	4.59 oz./acre	15.0
5	DPL 35B	3 foliar	2.2 oz./acre	6.0
		seed and	2.2 oz./50 lb.	
		3 foliar	2.2 oz./acre	26.0
		seed and	2.2 oz./50 lb.	
		3 foliar	4.4 oz./acre	33.0
6	Paymaster 1220BR	3 foliar	2.2 oz./acre	4.7
7	Paymaster 1220BR	3 foliar	2.2 oz./acre	13.1
		4 foliar	2.2 oz./acre	11.8
		seed and	2 oz./cwt	
		3 foliar	2.2 oz./acre	11.5
		seed and	2 oz./cwt	
		3 foliar	4.4 oz./acre	16.2
8	Stoneville BXN 47	3 foliar	2.2 oz./acre	49.6
		4 foliar	2.2 oz./acre	60.4
		seed and	2 oz./cwt	14.0
		3 foliar	2.2 oz./acre	
		seed and	2 oz./cwt	38.8
		3 foliar	4.4 oz./acre	

DPL varieties 20B (trial 1) and 33B (trial 2), and Paymaster 1220BR (trial 6) were treated with spray applications of Messenger[®] at 2.2 oz./acre starting at the first true leaf stage, followed by early bloom and mid bloom applications. Yields from treated plants were higher than for the untreated control plants of the same varieties with increases ranging from 4.7% to 11.4%. Messenger[®] was applied at rates of 2.2 and 4.4 oz./acre for a second trial on DPL 33B (trial 3) and at rates of 2.29 and 4.59 oz/acre on DPL 50B (trial 4). Spray applications for these two trials were at first true leaf, early bloom, and mid bloom. In both trials, the high rate applications gave higher yields than the lower rate applications. The dose/response effect of Messenger[®] on yield is strong evidence that the observed yield increases were a direct result of the Messenger[®] applications.

In trials on DPL 35B (trial 5), Stoneville BXN 47 (trial 8), and a second trial with Paymaster 1220BR (trial 7), multiple types of treatments were carried out including foliar spray and foliar spray combined with seed treatment, with varying numbers of applications and application rates. Foliar applications were made at rates of 2.2 oz./acre or 4.4 oz./acre and from three to four times during the season. Multiple foliar applications were also made in combination with seed treatment at 2 oz./cwt or 2.2 oz./50lbs. Foliar Messenger[®] applications were made beginning at first true leaf, followed by early bloom and mid bloom applications. All treatments gave increased yields over untreated control plants of the same varieties. In each of these three trials, low and high rate applications were made and effects on increased yield showed a dose response correspondence with the amount of Messenger[®] applied.

The average increase in yield for all applications in all eight trials was 19.6%. For those trials with low and high rate applications, the average yield increases were 14.6% for low rate applications and 25.8% for the high rate applications.

Example 2 - Increased Fruit Number in Transgenic Cotton Varieties Treated With Messenger[®]

Cotton yields can be directly impacted by the total number of bolls produced per plant. In three of the eight trials presented in Example 1 (trials 2, 6, and 7), analysis of the effects of Messenger[®] treatment on yield were extended to include

a comparison of the numbers of bolls produced by treated and untreated transgenic cotton. The results are summarized in Table 3 below.

Table 3: Increased Fruit Number in Messenger® Treated Transgenic Cotton

Trial	Cotton Variety	Treatment	Rate	Plant Mapping 1		Plant Mapping 2	
				Fruit per Plant	Percent Increase	Fruit per Plant	Percent Increase
2	DPL 33B	control	-	8.73	-	10.2	-
		3 foliar	2.2 oz./acre	10.78	23.5	11.0	7.8
6	Paymaster 1220BR	control	-	38.1	-	14.8	-
		3 foliar	2.2 oz./acre	42.1	10.4	17.6	18.9
7	Paymaster 1220BR	control	-	10.5	-	-	-
		3 foliar	2.2 oz./acre	11.1	5.7	-	-
		4 foliar	2.2 oz./acre	12.6	20.0	-	-
		seed and 3 foliar	2 oz./cwt 2.2 oz./acre	11.6	10.5	-	-
		seed and 3 foliar	2 oz./cwt 4.4 oz./acre	11.3	7.9	-	-
		3 foliar	4.4 oz./acre	-	-	-	-

In trials performed with DPL 33B (trial 2) and Paymaster 1220BR (trial 6), plants received three spray treatments with Messenger®. Applications were made as described in Example 1. Two boll counts were made in each trial, once after early bloom and a second time near harvest. A higher number of bolls was present on treated plants in both trial 2 and 6, at each of the early and late season plant mappings ranging from 7.8% to 23.5 % increase in number over control plants.

A second trial performed with Paymaster 1220BR (trial 7) was carried out with four types of treatments, as indicated in Table 3. A late season plant mapping revealed increased boll numbers for all Messenger® treated plants compared with untreated Paymaster 1220BR plants, with increases ranging from 5.7% to 20.0%.

The six Messenger® treatments in the two trials resulted in higher yields than obtained from untreated control plants. The results of these trials indicate that an increase in boll number can be a contributing factor to increased yields obtained from Messenger® treated cotton. There is an important distinction to be made between effects on yield from Messenger® and effects on yield resulting from transgenic traits conferred by insect or herbicide resistance genes such as those in the

transgenic cotton varieties in these trials. Such resistance genes do not increase the basic yield characteristics of the transgenic plant but simply reduce yield losses caused by insect or weed pressure. A combination of Messenger[®] and such resistance genes would have complementary effects on yield since Messenger[®] would provide a higher baseline yield through its effects on growth such as increased fruit number, while resistance genes such as Bt toxin would act to preserve that higher yield by reducing losses to insect pressure.

Example 3 - Increased Number of Open Bolls on Transgenic Cotton Treated With Messenger

The number of open bolls present at harvest is a factor in total yield. A trial including four different types of Messenger[®] treatments on the transgenic cotton variety Stoneville BXN 47 gave higher yields than obtained from untreated Stoneville BXN 47 (trial 8, Table 2). In addition to the measurements of overall yields, observations were extended to include a comparison of the numbers of open bolls at harvest on the Messenger[®] treated plants and untreated control plants. Four types of Messenger[®] treatments were tested in this trial. Two treatments consisted of either three or four foliar applications at rates of 2.2 oz./acre. The remaining two treatments consisted of seed application combined with foliar sprays using 2 oz/cwt for seed treatments and 2.2 or 4.4 oz./acre for three foliar applications. Open bolls were counted at three positions on the plants. Position 1 corresponded to the lowest node with bolls. Position 2 corresponded to the next node above on the stem. Position 3 included bolls at the third node and above combined into a single total. The totals for numbers of bolls at all three positions were also calculated. The results of this analysis are summarized in Table 4 below.

Table 4: Increased Number of Open Bolls From Messenger® Treated Transgenic Cotton

Trial	Cotton Variety	Treatment	Rate	Open Bolls			Per Plant	Percent Increase
				Position				
				1	2	3		
8	Stoneville BXN 47	control	-	3.56	0.33	0.00	3.89	-
		3 foliar	2.2 oz./acre	5.11	1.78	0.00	6.89	77.1
		4 foliar	2.2 oz./acre	5.45	1.45	0.11	7.00	79.9
		seed and 3 foliar	2 oz./cwt 2.2 oz./acre	5.44	1.22	0.00	6.67	71.5
		seed and 3 foliar	2 oz./cwt 4.4 oz./acre	5.22	2.00	0.11	7.33	88.4

The four types of Messenger® treatments performed in the trial resulted in increased numbers of open bolls on Stoneville BXN 47 relative to untreated Stoneville BXN 47. Increases in open bolls ranged from 71.5% to 88.4%. A dose response effect of Messenger® treatment on open boll number was evidenced by a higher percentage increase in open bolls with applications made at a rate of 4.4 oz./acre compared to applications made at 2.2 oz./acre.

Example 4 - Increased Yield from Transgenic Cotton Grown in a Field Infested with Reniform Nematodes.

Nematodes are parasitic worms that live in the soil and attack the roots of cotton. In an infested field, reniform nematodes can cause a 10-25% loss in yield and as much as 50% loss under stress conditions such as drought. A field trial to test effects of Messenger® treatment on cotton under nematode pressure was conducted in a field known to be infested with reniform nematodes. The cotton variety in the trial was Stoneville BXN 47, identified in Example 1. Since the bromoxynil transgene cannot provide resistance to nematodes, this cotton variety is just as susceptible to damage by nematodes as non-transgenic varieties.

Messenger® treatments of four types were applied in this trial. Two treatments consisted of either three or four foliar applications at rates of 2.2 oz./acre. The two other treatments consisted of seed application using 2 oz./cwt combined with three foliar sprays at rates of either 2.2 or 4.4 oz./acre. Foliar applications were made at first true leaf followed by early and mid bloom applications. Yields from treated

and untreated plots of Stoneville BXN 47 were determined as well as nematode populations in the soil from the plots. Results are summarized in Table 5 below.

Table 5: Increased Yield From Messenger® Treated Transgenic Cotton Grown in Nematode Infested Field

Trial	Cotton Variety	Treatment	Rate	Nematode Population		Percent Change	Yield Increase
				At Planting	At Harvest		
8	Stoneville BXN 47	control	-	9927	7609	- 23.4	-
		3 foliar	2.2 oz./acre	8889	6953	- 21.8	49.6
		4 foliar	2.2 oz./acre	8807	5948	- 32.5	60.4
		seed and 3 foliar	2 oz./cwt 2.2 oz./acre	6528	4867	- 25.4	14.0
		seed and 3 foliar	2 oz./cwt 4.4 oz./acre	10622	7957	- 25.1	38.8

Yields were substantially higher in all four plots receiving Messenger® treatment compared to untreated plots. The increased yields in response to Messenger® could be due to enhanced growth effects, induced resistance to nematodes, or a combination of both. Nematode populations declined over the course of the growing season with no significant difference in the amount of decline between treated and untreated plots, indicating that Messenger® did not directly affect nematodes. The significantly lower yield from the untreated Stoneville BXN 47 plot demonstrates the reserve potential for higher yield in a transgenic variety that can be elicited by Messenger®.

Example 5 - Application of Messenger® to Bt-transformed Corn Changes Toxicity Profile to Fall Armyworm

Non-Bt-transformed corn, (Yellow-sugary, 83-d maturity, cv. "Rogers", F1 Bonus, from Novartis) and Bt-transformed corn, (cv. "Rogers", GH-0937, also from Novartis) were planted in pots (one plant per pot, four replicate pots) and then placed in a greenhouse under normal conditions. When plants were 2-3 feet tall (pre-tassel), they were treated with a single foliar spray of Messenger® at a rate of 3 oz/acre in approximately 40 gal/acre. The concentration of harpin_{Ea} (active ingredient) in this spray was approximately 17ppm.

Five days after the application of Messenger[®], leaf discs of approximately 0.5 inch in diameter were collected from treated and non-treated plants and placed in on agar media in petri dishes. Fall armyworm (FAW, *Spodoptera frugiperda*) neonate larvae were added to each petri dish. Leaf discs were replaced as needed in order to provide a constant food supply to the larvae.

At 6 days after treatment (DAT), feeding activity by FAW was measured by counting the number of leaf disks completely eaten in both transformed and non-transformed corn, treated with and without Messenger[®]. As demonstrated in Table 6 below, substantial feeding activity occurred in both Messenger[®] and non-Messenger[®] treated, non-transformed corn. However, in Bt-transformed corn, very little feeding activity occurred.

Table 6 : Feeding Activity and Mortality Data for Fall Armyworm Feeding on Messenger[®] Treated and Non-treated Bt-corn and non-Bt-corn

Corn Description	Treatment	Feeding* 6 DAT	Mortality	
			7 DAT	8 DAT
Non-transformed	-	27	0%	0%
Non-transformed	Messenger [®]	34	0%	0%
Bt-transformed	-	2	30%	30%
Bt-transformed	Messenger [®]	0	80%	80%

* Number of leaf disks completely eaten by 20 larvae, with "0" indicating no leaf disks entirely eaten. DAT = days after treatment.

At 7 and 8 DAT no larval mortality was recorded in non-Bt-transformed corn, whether treated with Messenger[®] or not. However, in Bt-transformed corn, mortality at both 7 and 8 DAT was substantially lower for Messenger[®]-treated compared to non-Messenger[®]-treated (Table 6).

The increased mortality of FAW in Messenger[®]-treated, Bt-transformed corn suggests that application of Messenger[®] may have synergistic effects at controlling larval feeding activity.

Example 6 - Herbicide Resistant Transgenic Crops

A variety of technologies have been developed for production of transgenic plants resistant to herbicides including glyphosate, Synchrony, glufosinate, sethoxydim, imidazolinone, bromoxynil, and sulfonylurea. Each of these

technologies relies on the introduction of a single gene that confers resistance to a particular herbicide. Since the introduced gene is limited to a single function, other agronomically important traits of the crop plants remain unmodified. Glyphosate resistant transgenic cotton, soybean, and canola, for example, are susceptible to the same range of diseases that affect the non-transgenic parental lines from which the transgenic lines were developed. Yield losses due to disease could be minimized by combining genes for herbicide resistance and hypersensitive response elicitor expression in the same transgenic plant, thereby allowing the full benefits of the herbicide resistance trait to be realized.

Example 7 - Insect Resistant Transgenic Crops

Bt toxin protein from the soil bacterium *Bacillus thuringiensis* has been used on crops for many years as a topically applied insecticide with activity against specific classes of insects. A large number of genes have been isolated that encode different versions of the Bt toxin protein with varying specificities in insecticidal activity. The introduction of a gene encoding Bt toxin into potato was one of the first commercial applications of transgenic technology in crop plants. Since then, the commercialization of insect resistant crops expressing Bt toxin genes has been extended to include cotton and corn, with other crops under development. The specific insect resistance function of the Bt toxin gene is generally effective, but disease resistance and growth traits remain unaltered in the transgenic crops expressing Bt toxin genes. While yield losses due to insect pressure are reduced in Bt toxin expressing crops, they are still vulnerable to losses caused by pathogens. Bringing Bt toxin genes together with a transgene coding for hypersensitive response elicitor expression would produce crops that are resistant to pathogens as well as insects. An additional benefit would be increased yield due to the enhanced growth effect of the hypersensitive response elicitor.

Example 8 - Transgenic Crops With Enhanced Nutrient Value

Transgenic technology can be used to modify the balance of nutrients in crops to eliminate nutritional deficiencies. Some food crops are naturally deficient in particular amino acids that are a necessary component of the human diet. Cereal crops are often poor in tryptophan and lysine while vegetable crops and legume crops such as soybean are low in cysteine and methionine. Amino acids present at low amounts can be increased to nutritionally useful levels through the introduction of a gene encoding a protein with a high content of a particular amino acid that is normally lacking. Another approach that allows improvement of nutritional value is modification of an existing biochemical pathway or introduction of a novel biochemical pathway by introduction of a transgene. This can result in production of a compound with nutritional value that is normally absent or present in low amounts. Rice is an important food crop worldwide but is naturally low in vitamin A. Transgenic rice with increased vitamin A content could help to alleviate dietary deficiencies in this nutrient and is currently being developed. Transgenes can also be used to modify fatty acid biosynthesis pathways so as to produce food oils with altered levels of saturation. This method of improving nutritional value has been applied to canola, soybean, and flax so far. An aspect common to all the above approaches for enhanced nutritional quality is that improvements to the crops are limited to nutritional characteristics. Disease resistance and overall growth and yield properties of the crops remain unimproved. Combining a transgene coding for hypersensitive response elicitor expression with genes that confer enhanced nutritional value would allow the generation of transgenic crops that maximize the nutritional advantages through reduced losses to diseases and through improved yields due to enhanced growth.

Example 9 - Compensation For Transgenic Trait-Associated Losses In Yield

Introduction of a transgene for a beneficial trait can on occasion result in the introduction of a disadvantageous quality. For example, evidence indicates that the glyphosate resistance trait by itself can result in reduced yield in crops expressing

the resistance gene. A study done at the University of Wisconsin compared 1998 yields from glyphosate resistant soybean crops with yields from non-transgenic varieties at multiple sites in 8 Midwestern states and New York. At a majority of sites, yields of the glyphosate resistant soybeans were significantly lower than the non-transgenic varieties. The growth enhancement effect of a hypersensitive response elicitor could act to decrease or eliminate the yield penalty if combined with herbicide resistance genes in transgenic plants.

Introduction of a transgene may on occasion result in the loss of an advantageous trait. New Mexico State University reported losses to fungal infection in transgenic cotton varieties during the 1998 cotton season. Paymaster varieties that were insect resistant due to the presence of a Bt toxin transgene were susceptible to *Verticillium* wilt. Since the non-transgenic varieties had been resistant to *Verticillium* wilt, the introduction of the Bt toxin gene had resulted in loss of the fungal resistance trait. Negative side effects on disease resistance that might result from introduction of a transgene could be reduced or eliminated by combination with a transgene coding for hypersensitive response elicitor expression, which actively confers a broad range of disease resistance.

Example 10 - Pathogen Resistant Transgenic Crops

Crops are subject to attack by viral, bacterial, and fungal pathogens. An extensive amount research has been devoted to identifying ways to make crops resistant to pathogen attack. As a result, a growing number of genes have been identified that confer or have potential to confer pathogen resistance when expressed in transgenic plants. A major limitation of the resistance genes characterized so far is they have restricted ranges of effectiveness. A gene may confer resistance to viral but not fungal or bacterial pathogens, and *vice versa*. In many cases the protection is more narrowly limited to a small subset of viral, bacterial, or fungal pathogens. Transgenic plants expressing any of these resistance genes have reduced susceptibility to attack by specific pathogens or classes of pathogens, but the narrow range of resistance leaves the plants vulnerable to attack by many other pathogens. An example of how a narrow range of protection conferred by a transgene can leave a

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